The soil microbiota associated with New Zealand's kauri (*Agathis australis*) forests under threat from dieback disease.

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Alexa-Kate Byers

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Kauri (*Agathis australis*) is one of New Zealand's most iconic native tree species, which are of cultural significance to Māori and function as an ecological foundation species. However, only around 1% of the original kauri forest remains as a consequence of logging and clearance for agriculture. These remnant, fragmented kauri forests are now under threat from the spread of dieback disease caused by the soil-borne oomycete pathogen *Phytophthora agathidicida*, and urgent action is needed to manage the spread of dieback and secure the long-term health of kauri forests.

Soil microbiota are known to play a key role in supporting plant health and defending against soil-borne pathogens. Studying their response to disease outbreaks can guide the identification of disease suppressive microbial antagonists against pathogens such as *P. agathidicida*. In addition, investigating how disturbances impact the soil microbiota allows us to assess the indirect, secondary impacts of disturbances on forest ecosystems. Our knowledge on the microbial diversity of kauri forest soils and their responses to disturbances such as biological invasions is limited. The primary aim of this research was to characterise the taxonomic diversity of the kauri soil microbiota and their response to dieback, whilst screening members of the soil microbiota against *P. agathidicida* to test their potential to inhibit the pathogen. A secondary aim was to quantify differences in soil microbiota between kauri and adjacent pine (*Pinus radiata*) located at Waipoua Forest (Northland Region, New Zealand).

The findings of this study revealed that the establishment of pine significantly altered soil microbial community composition compared to that of old growth kauri forests, which included the loss of microbial taxa linked to plant health. This highlights the impact of historical disturbance on the soil environment surrounding kauri forests, which may impact their susceptibility to invading plant

pathogens. Significant differences were found in the diversity and composition of soil microbial communities associated with asymptomatic kauri compared to symptomatic kauri. Several microbial genera which have previously been reported to antagonise *Phytophthora* pathogens, such as *Penicillium*, *Trichoderma* and *Pseudomonas*, were found in higher abundance in asymptomatic kauri soils. These results may assist in the discovery of microbial taxa which enhance soil disease suppression. A supplementary kauri seedling infection study using soils from Waipoua Forest (Northland Region, New Zealand) identified that soil bacterial communities responded strongly to seedling infection. Using *in vitro* screenings, microbial strains belonging to the genera *Burkholderia*, *Paraburkholderia*, *Pseudomonas* and *Penicillium* that significantly inhibited the mycelial growth of *P. agathidicida* were identified. Their modes of inhibition, particularly for the *Burkholderia* strains, appear to be by the production of volatile and diffusible organic compounds.

This PhD thesis is the first study to have fully characterised the fungal and bacterial communities of old growth kauri soils using high throughput sequencing techniques. By doing so, this research has 1) assessed the impacts of forestry disturbance on the characteristics of the kauri soil microbiota 2) guided the identification of microbial taxa associated with potentially disease suppressive soils 3) highlighted the secondary impacts of dieback disease on the functional properties of the soil microbiota and 4) identified candidate microbial antagonists against *P. agathidicida*.

Keywords: *Phytophthora agathidicida*; *Agathis australis*; *Pinus radiata*; kauri dieback; soil microbiota; disease suppressive soils; microbial ecology; forest pathology; forest disturbance; high throughput sequencing; taxonomic diversity analysis; functional gene analysis; seedling infection study; phospholipid fatty acid (PLFA) analysis; *in vitro* screening; *Burkholderia*; *Penicillium*; head space solid-phase micro extraction-gas chromatography mass spectrometry (HS-SPME/GCMS) analysis; volatile organic compound (VOC); microbial antagonism.

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Chapter 1

Introduction

New Zealand's ancient kauri (*Agathis australis*) forests are under threat from the spread of kauri dieback, a root and collar rot disease caused by *Phytophthora agathidicida* (Beever et al., 2009; Weir et al., 2015). *P. agathidicida* is a highly virulent, soil-borne pathogen that is spread across kauri forests through the movement of contaminated soil, soil water and root pieces (Beever et al., 2009; Bellgard et al., 2013). Dieback disease is now widely distributed across most of the remaining natural geographic range of kauri in the upper North Island of New Zealand (Bradshaw et al., 2020; Weir et al., 2015). Given the threats posed to kauri from dieback disease, solutions are urgently required to manage its spread and secure the long-term health of kauri forests. Prior to kauri dieback being recognised as a key threat, kauri forests were already one of the most heavily disturbed indigenous forest types in New Zealand. Following European settlement in the early 19th century, kauri forests were subject to intense deforestation by uncontrolled logging for timber and land clearance for agricultural developments. Consequently, remnant primary kauri forests now cover less than 1% of their original area (Ecroyd, 1982; Ogden et al., 2001; Steward & Beveridge, 2010). Historical forestry disturbances and land use change have been proposed to influence pathogen spread by altering the environmental conditions and creating new transmission pathways across forest landscapes (Holdenrieder et al., 2004; Meentemeyer et al., 2008). Therefore, a key research theme of modern forest pathology is to understand how landscape level disturbances influence the spread of pathogens and disease outbreak (Cobb & Metz, 2017).

The role of the soil microbiota in influencing the spread of *P. agathidicida* and expression of kauri dieback is an understudied area of research. Nonetheless, it is an important research area as the soil microbiota have an important role in providing defence against soil pathogens, as well as supporting plant health and plant immune response in the face of pathogen attack (Baker & Cook, 1974; Berendsen et al., 2012). To address this knowledge gap, my PhD research aimed to characterise the taxonomic diversity of the kauri soil microbiota, whilst studying their response to dieback and identifying if any members exhibit antagonism to *P. agathidicida*. These aims were achieved by characterising the soil microbial communities associated with different kauri host states (i.e. infected *vs* uninfected). Following this, *in vitro* bioassays were performed to identify fungal and bacterial strains isolated from kauri forest soils that were able to inhibit *P. agathidicida*. In addition, by comparing differences in the soil microbiota between old growth kauri forest fragments and

adjacent pine (*Pinus radiata*) plantations, I assessed how historical forestry disturbances have impacted properties of the soil microbiota across Waipoua Forest (Northland Region, New Zealand).

1.1 Thesis outline

In my PhD thesis, I used a range of molecular and culture-based techniques to answer several fundamental questions regarding the soil microbial communities associated with kauri trees and their response to kauri dieback. The findings of this research are presented over the next six chapters. **Chapter 2** provides a brief literature review that summarises the contextual background of my PhD research. **Chapters 3** to **6** are presented as journal papers that have either been published or prepared for submission, however these chapters have been edited for the purposes of this thesis to avoid repetition across chapters. **Chapter 3** has been published in *FEMS Microbiology Ecology* and **Chapter 4** has been published in *Soil Biology and Biochemistry*. **Chapter 5** is scheduled for submission to *Forest Pathology* and **Chapter 6** is scheduled for submission to *Biological Control*. **Chapter 7** summarises the main findings and implications of my PhD research, including recommendations for further research.

1.1.1 Chapter 3

Chapter 3 aimed to identify differences in the diversity and composition of soil microbial communities associated with old growth kauri (*Agathis australis*) forest stands and adjacent pine (*Pinus radiata*) plantations. When studying the environmental factors that influence the spread of pathogens across forests, it is important to consider how anthropogenic disturbances have impacted forest health and whether these act as predisposing factors to disease outbreak (Cobb & Metz, 2017). Because of the functional roles that the soil microbiota plays in supporting plant health, studying how they have been impacted by forestry disturbances forms an important step in assessing how these disturbances may have influenced the susceptibility of forests to disease outbreaks. To assess how pine plantation establishment has impacted properties of the soil microbiota across Waipoua Forest (Northland Region, New Zealand), I used high throughput 16S rRNA and ITS gene region sequencing to characterise the microbial diversity and taxonomic composition of kauri and pine soils. In addition, the soil chemical properties (pH, total C, total N, C: N ratio, bioavailable N, Olsen P, organic matter content) of kauri and pine soils were measured to identify if soil abiotic properties were linked to differences in microbial community composition between kauri and pine soils.

Chapter 3 has been included in this thesis as a modified version of the following publication:

Byers, A.-K., Condron, L., Donavan, T., O'Callaghan, M., Patuawa, T., Waipara, N., & Black, A. (2020). Soil microbial diversity in adjacent forest systems- contrasting native, old growth kauri (*Agathis australis*) forest with exotic pine (*Pinus radiata*) plantation forest. *FEMS Microbiology Ecology*. https://doi.org/10.1093/femsec/fiaa047

1.1.2 Chapter 4

Chapter 4 aimed to identify differences in the diversity, composition and functional attributes of soil microbial communities associated with asymptomatic (i.e. not expressing symptoms of dieback disease) and symptomatic (i.e. expressing symptoms of dieback disease) kauri. Previous kauri dieback surveys have observed the presence of asymptomatic kauri in close proximity to symptomatic kauri (Beauchamp, 2013; Beever et al., 2009; Gadgil, 1974) and it has been hypothesised that additional environmental factors may influence pathogen induced disease expression. By studying the differences in soil microbial diversity and community composition between asymptomatic and symptomatic host states, I aimed to identify if there were properties of the soil microbiota that may be associated with disease suppression. For **Chapter 4**, I used high throughput 16S rRNA and ITS gene region sequencing to characterise the soil microbial communities associated with asymptomatic and symptomatic kauri. In addition, a functional gene array (GeoChip 5S) was used to identify differences in the abundance of microbial genes related to carbon (C) and nitrogen (N) cycling between asymptomatic and symptomatic soils. The aim of this was to identify how dieback disease has impacted soil microbial function, information which will help us evaluate the long-term impacts of kauri dieback on forest soil health.

Chapter 4 has been included in this thesis as a modified version of the following publication:

Byers, A.K., Condron, L., O'Callaghan, M., Waipara, N., & Black, A. (2020). Soil microbial community restructuring and functional changes in ancient kauri (*Agathis australis*) forests impacted by the invasive pathogen *Phytophthora agathidicida*. *Soil Biology and Biochemistry, 150*, 108016. https://doi.org/10.1016/j.soilbio.2020.108016

1.1.3 Chapter 5

Chapter 5 aimed to identify differences in the diversity, composition and biomass of soil microbial communities associated with *P. agathidicida* inoculated and non-inoculated kauri seedlings. The purpose of this was to build on the findings of **Chapter 4** by using a seedling infection study to characterise the response of the soil microbiota to kauri seedling infection in a controlled environment. This allowed for several factors to be standardised which could not be standardised in

Chapter 4, such as the inoculum load of *P. agathidicida* applied to each seedling, seedling age, the time of initial infection and the surrounding environmental conditions (e.g. light, water, temperature). Using high throughput 16S rRNA and ITS gene region sequencing, the differences in microbial diversity and taxonomic composition of inoculated and non-inoculated seedlings were characterised. Additionally, phospholipid fatty acid (PLFA) analysis was used to identify differences in the microbial biomass of soils from inoculated and non-inoculated seedlings.

Chapter 5 has been included in this thesis as a draft paper that will be submitted to *Forest Pathology*.

1.1.4 Chapter 6

Chapter 6 aimed to identify fungal and bacterial strains isolated from kauri soils that were able to inhibit the growth of *P. agathidicida in vitro*. This was so we could begin to select candidate microbial antagonists against *P. agathidicida* which could potentially be used for the future management of kauri dieback. To do this, I used culture-based bioassays to screen fungal and bacterial strains isolated from kauri forest soils against *P. agathidicida* and identify those which were able to inhibit the mycelial growth of *P. agathidicida*. In addition, the volatile organic compounds (VOCs) released by the microbial strains were characterised using head space solid phase micro extraction (HS- SPME) and gas chromatography mass spectrometry (GC-MS) analysis. The aim of this was to identify VOCs released by the microbial strains that may be associated with the inhibition of *P. agathidicida*.

Chapter 6 has been included in this thesis as a draft paper that will be submitted to *Biological Control.*

1.1.1 Study site- Waipoua Forest (Northland Region, New Zealand).

Kauri soil samples required for each of the four objectives were sampled from Waipoua Forest Sanctuary and Waipoua Kauri Management and Research Area, hereafter referred to as 'Waipoua Forest'. Waipoua Forest is a 13 000 hectare conservation area located on the west coast of Northland, New Zealand occurring at 35° 38'S latitude and 173° 34'E longitude (Figure 1.1) (Burns & Leathwick, 1996). The Waipoua region has a hilly and gently sloping landscape formed of low fertility clay soils which overlay Waipoua Basalt as the main geological substrate (Burns, 1995; Burns & Leathwick, 1996). Waipoua Forest was declared a forest sanctuary in 1952 and contains the largest remaining tracts of old growth kauri forests left in New Zealand. Additionally, Waipoua Forest is home to the iconic *Tāne Mahuta* and *Te Matua Ngahere*, which are the largest and oldest extant

kauri (Burns & Leathwick, 1996; Steward & Beveridge, 2010). Across Waipoua Forest there are extensive pine (*Pinus* spp.) plantations (Ogden et al., 2001; Thode, 1983) that cover an area of approximately 3400 hectares (http://teroroa.iwi.nz/forestry.html). Historically, the kauri forests of Waipoua Forest have been subject to disturbances from logging, gum collection, fire clearance, silviculture, exotic forestry development, experimental plantings of introduced kauri and the construction of State Highway 12 (Beachman, 2017; Steward & Beveridge, 2010; Thode, 1983). P. agathidicida has previously been considered an introduced pathogen to New Zealand (Weir et al., 2015). Although the primary pathway that introduced *P. agathidicida* to New Zealand has not been identified, Waipoua Forest is considered one of the original introduction sites of the pathogen (Beachman, 2017).

Figure 1.1. The location of Waipoua Forest on the west coast of the Northland Region, New Zealand. Map data; Google Earth 2020.

Therefore, Waipoua Forest was considered the most suitable site for the aims of my PhD research because of the area's coverage of remnant old growth kauri forests, widespread distribution of *P. agathidicida*, and establishment of exotic pine plantations. A preliminary survey was conducted across Waipoua Forest to assess the distribution and access of symptomatic kauri, asymptomatic

kauri and pine plantations prior to undertaking soil sampling (Figure 1.2). This was guided using a survey conducted by the Department of Conversation (DoC) in 2011 (Tom Donovan, personal communications, April 2018) which mapped the locations of kauri whose soil samples had tested positive or negative for *P. agathidicida*.

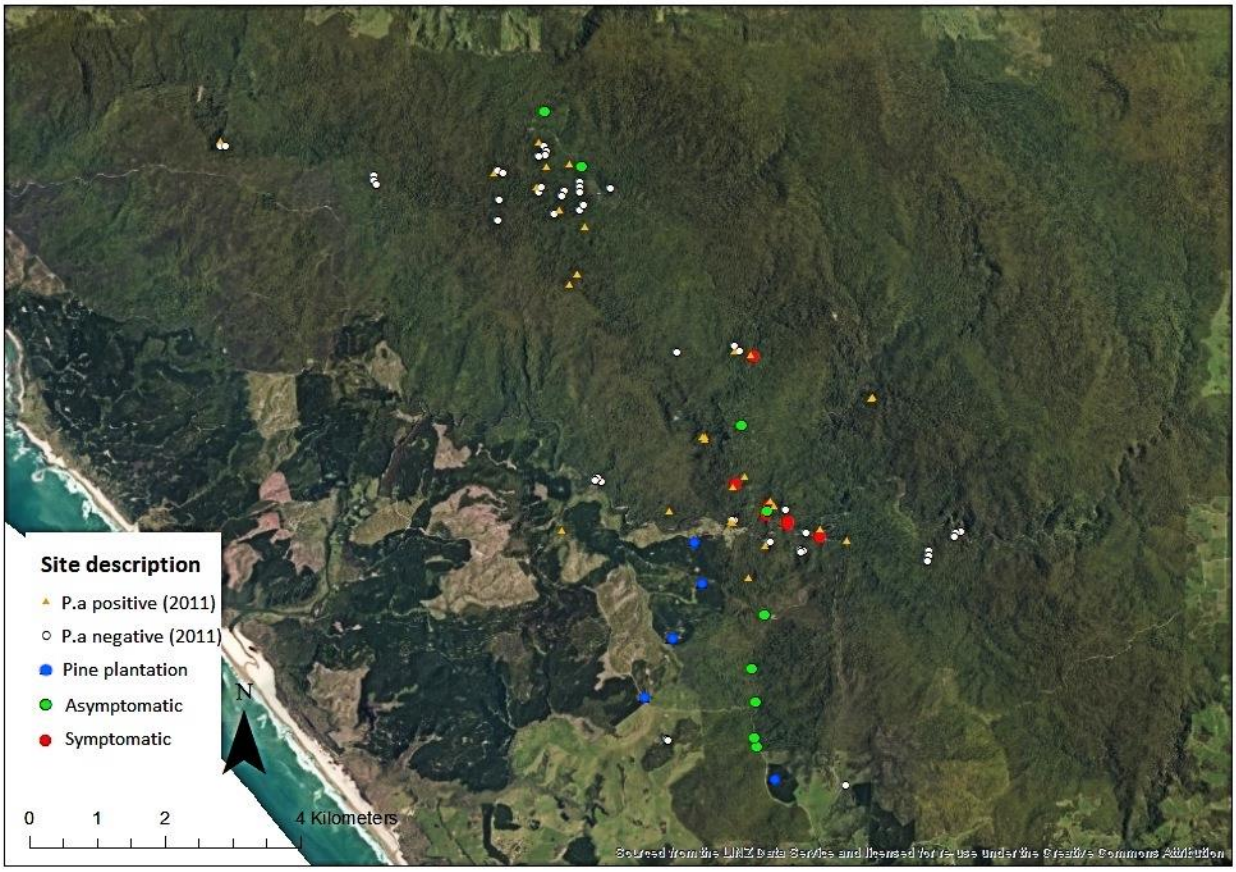

Figure 1.2. The presence and distribution of asymptomatic kauri, symptomatic kauri and pine plantations across Waipoua Forest (April, 2018). Also shown are the locations of kauri whose soil samples tested positive or negative for *P. agathidicida* in a survey conducted by the Department of Conversation (DoC) in 2011. Background map data imagery; Google Earth 2020.

Chapter 2

Literature and background

2.1 The ecological and cultural significance of kauri forests

Kauri are New Zealand's only endemic member of the *Araucariaceae*, an ancient family of coniferous trees that date back to the Triassic period (Steward & Beveridge, 2010). Kauri are one of the largest living conifers worldwide and form a huge presence in their forests, having diameters of up to 5 m, heights of up to 50 m and ages of up to 1700 years (Ahmed & Ogden, 1987; Ecroyd, 1982; Steward & Beveridge, 2010). The dominance and influence of kauri means their decline would impact kauri forest dynamics and ecosystem processes greater than the loss of any other plant species (Wyse et al., 2014). Kauri function as a foundation species within their forests and significantly influence carbon and nitrogen cycling, as well as the properties of their surrounding soil and plant environment (Macinnis-Ng & Schwendenmann, 2015; Verkaik et al., 2006; Wyse et al., 2014). Additionally, the decline of kauri would be an immense cultural loss to New Zealand as kauri are recognised as a taonga species and regarded as living ancestors by the indigenous Māori (Black et al., 2018; Nuttall et al., 2010). Most of the remnant ancient kauri forests occur in the Northland region of New Zealand where they form an integral component of the cultural and spiritual identity of the local Māori tribes across the region (Lambert et al., 2018).

Kauri forests are naturally distributed across the warm climatic zones of the upper North Island, occurring north of latitude 38^{o} S (Steward & Beveridge, 2010). Kauri are pioneers on infertile soils and over their long lives they further reduce soil nutrient availability, forming highly acidic (typically pH 4) podzolised soils (Jongkind et al., 2007; Wyse, 2012). The large litter outputs and slow decomposition rate of kauri leaf litter causes a thick mor-humus layer to form beneath mature kauri which can be up to 3 m deep (Steward & Beveridge, 2010). In addition, the high tannin content of kauri leaf litter leads to the build-up of large amounts of nitrogen (0.65 kg N/m^2) in the soil organic layer (Verkaik et al., 2006; Wyse et al., 2014). The slow decomposition rate and high proportion of woody material making up kauri litter means that kauri soils are incredibly carbon dense, having stores of up to 670 Mg C ha^{-1} (Macinnis-Ng & Schwendenmann, 2015; Schwendenmann & Michalzik, 2019; Silvester & Orchard, 1999). Kauri play a key role in shaping the composition of their surrounding vegetation, supporting one of the most species rich forest types in New Zealand (Ogden et al., 2001; Wardle, 1991; Wyse et al., 2014).)The acidic, low fertility soils associated with kauri forests selectively filters the surrounding vegetation of kauri, with co-occurring plant species

including neinei (*Dracophyllum latifolium*), tanekaha (*Phyllocladus trichomanoides*), totara (*Podocarpus totara*) and rimu (*Dacrydium cupressinum*) (Steward & Beveridge, 2010; Wyse et al., 2014).

2.2 Kauri dieback

2.2.1 *Phytophthora agathidicida*

Phytophthora agathidicida is recognised as a highly virulent pathogen of kauri and the primary causal agent of kauri dieback in New Zealand (Beever et al., 2009; Weir et al., 2015). Members of the *Phytophthora* genus are highly destructive plant pathogens in both agricultural (e.g. *Phytophthora infestans* and potato late blight, Ireland) and natural (e.g. *Phytophthora ramorum* and sudden oak death, North America) ecosystems worldwide (Fry & Goodwin, 1997; Rizzo et al., 2002; Thines, 2013). The *Phytophthora* genus belongs to the Oomycetes, which although share similar nutritional modes and ecological roles with true fungi, are more closely related to diatoms and brown algae (Kingdom: Stramenopila) (Beakes et al., 2012; Thines, 2013). Phylogenetically, species belonging to the *Phytophthora* genus can be split into 10 different clades, with *P. agathidicida* belonging to the poorly studied Clade 5 alongside *P. castaneae*, *P. heveae* and *P. cocois* (Weir et al., 2015). Previously, *P. agathidicida* has been assumed an introduced pathogen to New Zealand, and was suggested to have originated from East Asia or the Pacific based on the origins of other Clade 5 *Phytophthora* species (Weir et al., 2015). However, recent mitochondrial genome evidence has detected that *P. agathidicida* may have been present in New Zealand for several hundred to several thousand years (Winkworth et al., 2021).

2.2.2 The discovery of kauri dieback

Kauri dieback was first reported to be afflicting kauri on Great Barrier Island (Hauraki Gulf, Auckland) in the 1970s. Upon discovery, the causal agent of kauri dieback was originally misidentified as *Phytophthora heveae* (Gadgil, 1974) and the disease attracted limited interest. In 2006, attention to the disease increased as dieback was observed on mainland kauri forests in the Waitakere Ranges, Auckland (Beever et al., 2009). Following this, the causal agent of dieback was identified as a new *Phytophthora* species that was given the working name *Phytophthora* 'taxon *Agathis*' and was later formally classified as *Phytophthora agathidicida* by Weir et al. (2015). The pathway by which *P. agathidicida* entered New Zealand is unknown. However, Waipoua Forest has been proposed as one of the main sites of introduction, with the pathogen possibly brought in with contaminated foreign plant and soil material to seedling nurseries in the 1940/50s (Beachman, 2017). Furthermore, there is evidence to suggest that *P. agathidicida* spread from contaminated kauri nurseries located within

Waipoua Forest to multiple other forest sites in the 1950s (Beauchamp & Waipara, 2014). Logging activities within Waipoua Forest during this time have been proposed to have exacerbated the spread of dieback in the area (Beachman, 2017).

2.2.3 The distribution and spread of kauri dieback

Kauri dieback is reported to be distributed across most of the remaining natural geographic range of kauri (Figure 2.1) with disease expression and tree mortality affecting all age and size classes (Bellgard et al., 2016). Previous surveillance has found four main forest regions to be disease foci: Waipoua Forest, Northland; Waitakere Ranges, Auckland; Punaruku, Northland; and Great Barrier Island, Hauraki Gulf Auckland (Beauchamp, 2013; Waipara et al., 2013). *P. agathidicida* is spread across kauri forests via the trafficking of infested soil, soil water and root material by human activity (Beever et al., 2009; Bellgard et al., 2013) and, to a lesser extent, feral pig and cattle movement (Bassett et al., 2017; Beever et al., 2009; Pau'uvale et al., 2011). Observational data has estimated *P. agathidicida* to spread through infected kauri forests at a rate of 3 m per year, a similar rate of spread to *Phytophthora cinnamomi* mediated diseases across south-western Australia (Beever et al., 2009).

The reproductive cycle of *P. agathidicida* has several different life stages including the production of sexual oospores, asexual sporangia and zoospores, and vegetative hyphal growth (Bellgard et al., 2016; Bradshaw et al., 2020; Weir et al., 2015). Under favourable environmental conditions, such as poorly drained or flooded soils, the dormant and highly resistant oospores germinate to produce sporangia. These sporangia release motile zoospores that swim towards the plant roots through water filled pores in the soil. Upon contact, zoospores encyst and produce penetrative hyphae that colonise the root. Over time, *Phytophthora* colonises and parasitizes the plant root system until it reaches the tree collar where expression of above-ground symptoms can be observed (Bellgard et al., 2016; Bradshaw et al., 2020). As shown in Figure 2.2, symptoms of kauri dieback can include root and collar rot, severe chlorosis, defoliation, lower trunk gummosis and tree mortality (Waipara et al., 2013). The latency period for kauri dieback, which is the time between initial root infection and onset of tree disease expression, is not yet known. Additionally, the time period between onset of disease symptoms and eventual tree mortality is highly variable and can range from 1 to 10 years (Bradshaw et al., 2020).

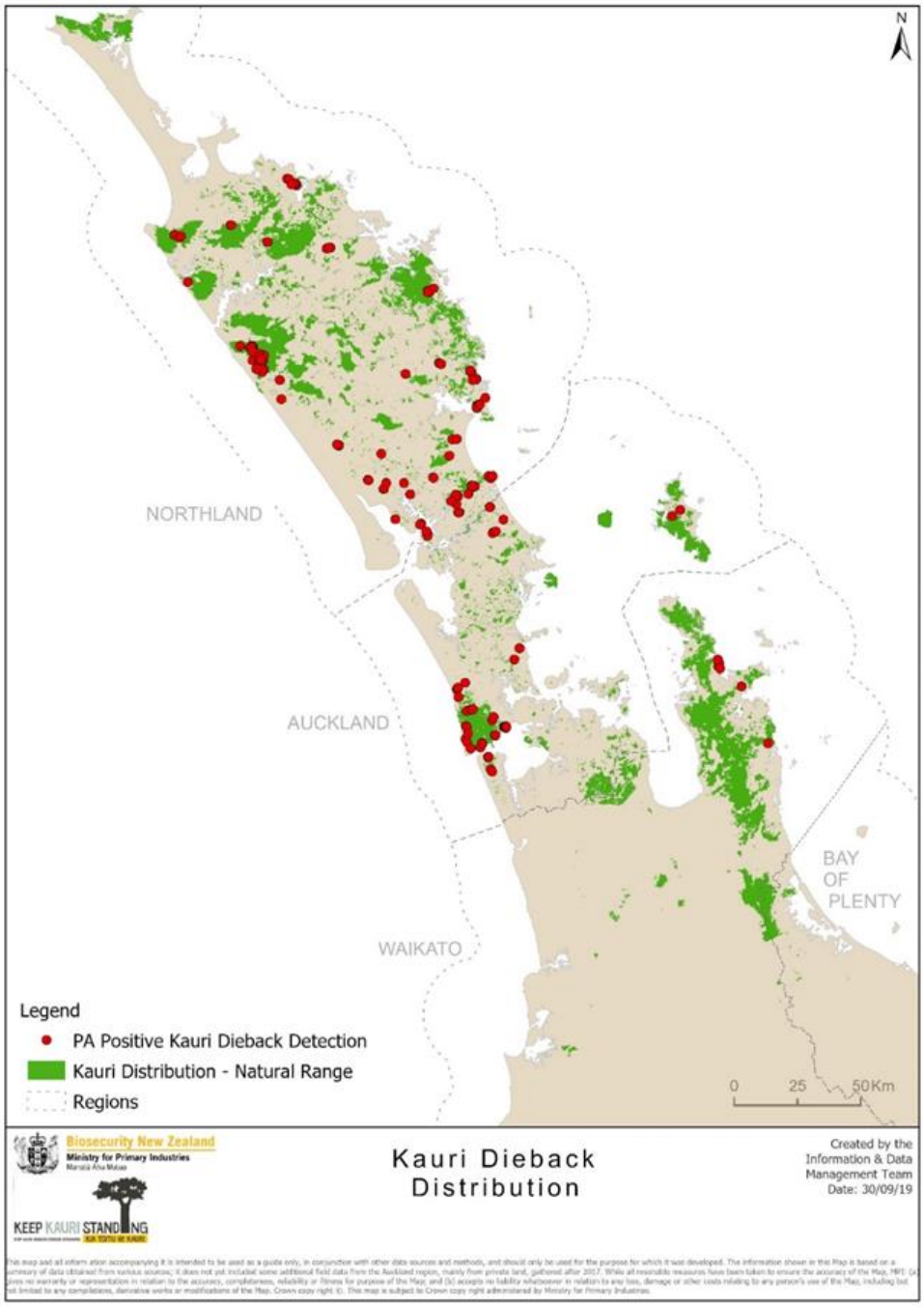

Figure 2.1. The distribution of kauri forest soil samples that tested positive for *P. agathidicida* across natural range of kauri along the upper North Island of New Zealand. Map retrieved from the Kauri Dieback Programme (https://www.kauridieback.co.nz/kauri-maps/) and created by Biosecurity New Zealand (Ministry for Primary Industries (MPI), Crown copy right ©).

Figure 2.2. Symptoms of kauri dieback including tree defoliation, lower trunk gummosis, and 'stag head' branch structures that were observed at Waipoua Forest (Northland Region, New Zealand). Images credits; Alexa Byers, April 2018.

2.2.4 Management of kauri dieback

In 2009, the Kauri Dieback Programme (KDP) (https://www.kauridieback.co.nz) was established in response to the increasing threat posed to remnant kauri forests from dieback. The KDP is a partnership programme between Biosecurity New Zealand (MPI), the Department of Conservation (DoC), Auckland Council, Waikato Regional Council, Northland Regional Council, Bay of Plenty Regional Council, Te Roroa, Tangata Whenua Roopu and several other independent research bodies (KDP, 2020a). Managing kauri dieback is a huge challenge because *Phytophthora* pathogens are almost impossible to fully eradicate once established (Scott & Williams, 2014). There are many knowledge gaps in kauri dieback research which limit progress in managing the disease (Black & Dickie, 2016; Bradshaw et al., 2020). Current management practices are primarily focused on containment and preventing the spread of infected soil to new forest areas, such as the implementation of track closures and controlled area notices (KDP, 2020b).

Several standardised methods have been developed to detect *P. agathidicida* in forest soils, such as a lupin baited soil bioassay (Bellgard et al., 2013), a TaqMan real-time PCR assay (Than et al., 2013) and a loop-mediated isothermal amplification (LAMP) assay (Winkworth et al., 2020). Despite the advances in molecular diagnostic tools for *P. agathidicida*, the soil baiting bioassay is still the most routinely used. One benefit of the soil baiting bioassay is that it confirms the presence of only the viable and infectious propagules of *P. agathidicida* (Bradshaw et al., 2020). However, completing one round of soil baiting is a long process that can take up to 4 weeks to get accurate diagnostic results. This greatly limits the speed at which management can respond to the spread of the pathogen and is not conducive for effective disease containment.

Previous research aiming to find solutions to kauri dieback has focused on tree phosphite injections (Horner & Hough, 2013), oospore deactivation methods (Dick & Kimberley, 2012), genetic resistance screenings (Herewini et al., 2018) and discovery of native plant root exudates with anti-*Phytophthora* activities (Lawrence et al., 2017; Lawrence et al., 2019). There is currently no published research on the potential for chemical control to be used to manage kauri dieback, however there are several research projects underway in this area (KDP, 2020b). One understudied research area is how the abiotic and biotic environmental factors of kauri forests, such as soil microbial community composition and soil physicochemical properties, impact the spread of dieback disease (Bradshaw et al., 2020). Using *in vitro* growth response assays, Lewis et al. (2019) identified that the oospore production of *P. agathidicida* was significantly higher when cultures were incubated in pine forest soils compared to kauri forest soils collected from Waipoua Forest (Northland Region, New Zealand). However, it is important to note that none of the soil samples collected directly from pine forests tested positive for the presence of *P. agathidicida* and this theory has not been validated in the field.

2.3 The soil microbiota

The soil microbiota has numerous functional roles in supporting plant health through plant nutrient acquisition, mitigation against abiotic stressors, induction of plant systemic resistance and defence against pathogens (Andreote & Pereira e Silva, 2017; Trivedi et al., 2020). Large tree species with long life histories have a limited ability to modulate their fitness against emerging pathogens (Desprez-Loustau et al., 2015). However, root exudates released by plants under attack can stimulate and enrich soil microorganisms that are better able to modulate their activity to outcompete, antagonise or hyper parasitize invading pathogens (Desprez-Loustau et al., 2015; Raaijmakers et al., 2009; Trivedi et al., 2020). This form of plant defence against pathogens, provided by the activities of the soil microbiota, can result in the formation of disease suppressive soils.

Disease suppressive soils provide a microbially mediated form of defence against plant pathogens by either preventing pathogen establishment in soils or by allowing pathogens to establish without causing plant disease expression (Baker & Cook, 1974). Disease suppressive soils can be split into two forms- general and specific. However many soils can possess features of both, with specific disease suppression often being supported by a basal level of general disease suppression (Weller et al., 2002).

General disease suppression is a community based form of defence whereby the total soil microbial community collectively prevents pathogen establishment by competing for limited resources in the soil environment (Schlatter et al., 2017; Weller et al., 2002). The levels of general disease suppression in soils can be enhanced using soil amendments (e.g. organic matter) which can increase the diversity, biomass and activity of resident soil microbiota (Bonilla et al., 2012; Rosskopf et al., 2016). In contrast, specific disease suppression is a targeted form of defence whereby certain members of the soil microbiota antagonise specific life stage of the invading pathogen (Schlatter et al., 2017). Following plant disease outbreak, specific disease suppression can be induced into soils following the enrichment of microorganisms with antagonistic traits towards the pathogen (Raaijmakers & Mazzola, 2016). Forms of microbial antagonism against pathogens include the release of anti-microbial secondary metabolites and lytic enzymes, effector mediated reductions in pathogen virulence and direct pathogen hyper-parasitism (Raaijmakers et al., 2009). In addition, microorganisms can enable plants to suppress disease by stimulating the plant immune responses via induced systemic resistance (ISR) (Berendsen et al., 2018; Pieterse et al., 2014). Additionally, specific disease suppression can be purposefully induced in soils by transferring a small volume of suppressive soil, or an inoculum load of the microbial agent(s) responsible for disease suppression (Mendes et al., 2011; Weller et al., 2002). Previous attempts at inducing disease suppression into soils has largely failed when tested under field environments. Research commonly focuses on targeting a single species responsible for suppression, however specific disease suppression is often attributed to the activities of a suite of soil microorganisms (Expósito et al., 2017; Raaijmakers & Mazzola, 2016). Therefore, when studying disease suppressive soils it is important to identify how the diversity and functional traits of the whole soil microbiota collectively support plant health.

2.4 What is already known about the kauri soil microbiota?

There have been a limited number of studies conducted on the microbial associates of kauri, with most of these studies being focused on the arbuscular mycorrhizal fungi (AMF) fungi which colonise kauri roots (Bradford, 2020; Han, 2016; Morrison & English, 1967; Padamsee et al., 2016). Morrison and English (1967) Proposed that the colonisation of kauri root nodules by arbuscular mycorrhizal

fungi (AMF) aids root uptake of phosphorus, a nutrient which commonly has a limited bioavailability in kauri soils. Using high throughput sequencing, Padamsee et al. (2016) identified that the AMF associates of kauri roots and nodules belonged to multiple lineages of Glomeromycota. PhD research by Han (2016) identified differences in the structure of AMF communities associated with asymptomatic and symptomatic kauri roots. In addition, this research observed the presence of dark septate endophytes within kauri roots which should be of interest for further studies examining their relationship with *P. agathidicida*. Furthermore, MSc research by Bradford (2020) identified two fungal endophytes belonging to the classes Sordariomycetes and Leotiomycetes that demonstrated an antagonistic relationship with *P. agathidicida in vitro*.

Kauri have been found to associate with endomycorrhizal and saprophytic fungi, however they have not been observed to associate with ectomycorrhizal fungi (McKenzie et al., 2002). Most of the fungi reported on kauri by McKenzie et al. (2002) were saprobes found on decaying plant material, however the same study noted that the acidic soil environment which forms beneath kauri cannot support a prolific number of saprophytic fungi. Nonetheless, McKenzie et al. (2002) reported 189 identified species and 75 unidentified species to associate with kauri based on the findings of non-targeted surveys and culture collections. Twelve of these species were recorded as locally endemic to kauri forests, which included *Helotium allantosporum, Corticium kauri, Hymenochaete agathicola, Chaetopsis prosbosciphora, Chalara agathidis, C. constricta, C. scabrida, C. stipitata* and *Ulocoryphus mastigophorus*.

Despite this previous research, which has mainly focused on the AMF associates of kauri roots, our knowledge on the fungal and bacterial communities associated with kauri forest soils is limited. Before we can study how the soil microbiota influences the spread and expression of kauri dieback, we need to comprehensively characterise the taxonomic diversity of the kauri soil microbiota at the community level.

2.5 Methods to characterise the soil microbiota

As discussed in **Section 2.3**, disease suppression can be induced in soils following disease outbreak by the enrichment of soil microorganisms with antagonistic traits towards the pathogen (Raaijmakers & Mazzola, 2016). Therefore, by studying the response of the soil microbiota to disease outbreak, we can identify microorganisms that have potential to suppress plant infection and disease expression (Galiana et al., 2011). The methods used to characterise the diversity, structure and function of the soil microbiota can be broadly split into two forms- culture dependent (Islam et

al., 2016; Syed-Ab-Rahman et al., 2018) and culture independent (Mendes et al., 2011; Penton et al., 2014).

2.5.1 Culture dependent methods

When aiming to identify soil microorganisms responsible for disease suppression, culture dependent studies often begin with the isolation of microbial strains from field soils associated with a plant disease outbreak or from soils that display disease suppressive properties. Upon isolation, several forms of culture-based experiments may be performed to identify microbial strains that inhibit the growth, or a specific life stage, of the pathogen of interest (Zohara et al., 2016). Following the demonstration of microbial antagonism *in vitro*, *in vivo* studies may be used to assess the ability of microorganisms to suppress pathogen growth or plant disease expression in pathogen infested soils (Syed-Ab-Rahman et al., 2018; Widmer, 2014). Culture based studies have long been an essential method of discovering, obtaining and demonstrating the use of microorganisms as effective control agents. Previous culture based studies have successfully isolated fungal and bacterial strains that can antagonise *Phytophthora* pathogens (Caulier et al., 2018; Ma et al., 2008; Zohara et al., 2016). For example, Zohara et al. (2016) isolated novel *Pseudomonas* strains that were shown to inhibit mycelial growth, sporangia production and zoospore release of *Phytophthora capsici*. Méndez-Bravo et al. (2018) used culture bioassays and solid-phase microextraction coupled with gas chromatography-mass spectrometry (SPME/GC-MS) to identify bacterial strains that inhibited the growth of *Phytophthora cinnamomi* through production of volatile organic compounds (VOCs).

There are several disadvantages to using culture dependent methods to study disease suppressive soils. Firstly, only a small percentage (1-10%) of soil microorganisms can be cultured in a laboratory environment which limits the collection of microorganisms that may be contributing to disease suppression (Gurusinghe et al., 2019; McCaig et al., 2001). Furthermore, microbial antagonists often fail to confer disease suppression when tested in the field. This is because they cannot establish and elicit the functional traits required to provide disease suppression when introduced into the rhizosphere, particularly when competing with the resident soil microbiota (Alabouvette et al., 2009; Expósito et al., 2017). When searching for disease suppressive microbial antagonists against a specific soil-borne pathogen, it is preferential to screen the resident soil microbiota associated with the plant host under investigation. This is because introducing or amending the population levels of resident soil microorganism's provides a greater chance of them being able to establish and express their disease suppressive functional traits (Raaijmakers & Mazzola, 2016; Zohara et al., 2016).

2.5.2 Culture independent methods

In contrast, culture independent methods use molecular tools to understand how the diversity, structure and function of the soil microbiota contributes to disease suppression. Profiling the taxonomic diversity of microbial communities can use methods such as T-RFLP (Donn et al., 2014), DGGE (Hjort et al., 2007), qPCR (Kyselková et al., 2009), 16S rRNA and ITS gene region high throughput sequencing (Hannula et al., 2020) and functional gene arrays (He et al., 2007; Mendes et al., 2011). Modern advances in the speed, affordability and volume of data provided by genomic sequencing has improved our ability to characterise the soil microbiota and identify the taxonomic groups responsible for disease suppression (Blaya et al., 2016; Mendes et al., 2011). Performing high throughput sequencing on targeted gene regions, such as the bacterial 16S rRNA and fungal ITS gene region, is a commonly used method that provides a semi quantitative analyses on the diversity and structure of microbial communities (Lauber et al., 2009; Lindahl et al., 2013; Schmidt et al., 2013). For example, Siegel-Hertz et al. (2018) used 16S rRNA and ITS gene region sequencing to compare the microbial profiles of field soils suppressive and conductive to *Fusarium* wilt. By comparing their microbial profiles, Siegel-Hertz et al. (2018) identified several bacterial and fungal genera that were exclusive to or found at a higher abundance in disease suppressive field soils. Compared with culture dependent methods, profiling the diversity of the soil microbiota using culture independent methods can identify previously undetected or uncultured microorganisms associated with soil disease suppression. However, many of these methods do not provide information on the ecological functions of soil microorganisms, nor how they interact with pathogens or plants to suppress disease (Expósito et al., 2017).

More recently, meta-omics technologies such as metagenomics (Carrión et al., 2019), metatranscriptomics (Chapelle et al., 2016), metaproteomics (van der Voort et al., 2016) and metabolomics (Hayden et al., 2019) have gained greater research interest. Meta-omics can provide a systems level understanding on how the diversity, gene expression, protein signalling and metabolic signalling of the soil microbiota contributes to disease suppression (Allen White et al., 2017). For example, Chapelle et al. (2016) used metatranscriptomics to identify bacterial families that upregulated their expression of stress related genes following invasion of the pathogen *Rhizoctonia solani*. Findings revealed that the taxonomic identity and functional traits of select bacterial families were responsible for disease suppression. A study by Hayden et al. (2019) used metabolomics to compare the biochemical profiles of soils suppressive and conductive to *Rhizoctonia solani.* Findings identified an anti-microbial metabolite which can now serve as a functional biomarker for *R. solani* disease suppressive soils. Meta-omics technologies offer exciting opportunities to link the collective function of the soil microbiota to their roles in soil disease suppression. However, there are several

limitations preventing the widespread application of meta-omics technologies, primarily their lack of standardised methodological, computational, and analytical pipelines (Segata et al., 2013). Thus, for the purpose of this PhD thesis, I used high throughput 16s rRNA and ITS gene region sequencing to analyse the kauri soil microbiota as the current protocols and pipelines are more established which enables the results to be compared with future studies.

2.6 Conclusion

The combined threats of historical anthropogenic disturbance and kauri dieback threaten the long-term health and survival of New Zealand's ancient kauri forests. Our knowledge on how the kauri soil microbiota respond to these threats is an understudied area of research. In fact, the soil microbiota associated with kauri has yet to be fully characterised, with most of the scientific knowledge based on targeted surveys and culture collections. Such information is important to understand, as the soil microbiota provide multiple roles in supporting plant health and defending against soil pathogens. Modern advances community profiling using NGS (i.e. 16S rRNA and ITS gene region sequencing) has popularised their use in studies examining how the taxonomic diversity of the soil microbiota relates to plant health and soil disease suppression. In addition, culture-based techniques provide an established method of studying how specific members of the soil microbiota interact with target pathogens to suppress disease. Therefore, this PhD thesis used molecular and culture-based techniques to study the soil microbiota associated with kauri forests afflicted with dieback disease. This allowed us to characterise the taxonomic diversity of the kauri soil microbiota and their response to dieback, whilst screenings members of the soil microbiota against *P. agathidicida* to test their potential to inhibit the pathogen.

Chapter 3

Soil microbial diversity in adjacent forest systems- contrasting native, old growth kauri (*Agathis australis*) with forest with exotic pine (*Pinus radiata*) plantation forest.

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Supplementary material for this chapter is provided in Appendix A.

3.1 Abstract

Globally, the conversion of primary forests to plantations and agricultural landscapes is a common land use change. Kauri (*Agathis australis*) are one of the most heavily impacted indigenous tree species of New Zealand. Currently, less than 1% of primary kauri forest remains as fragments adjacent to pastoral farming and exotic forest plantations. By contrasting two forest systems, this research investigated if the fragmentation of kauri forests and establishment of pine plantations (*Pinus radiata*) has significantly impacted the diversity and composition of soil microbial communities across Waipoua Forest (Northland Region, New Zealand). Using high throughput 16S rRNA and ITS gene region sequencing, these findings identified that fungal and bacterial community composition significantly differed between kauri and pine forest soils, with fungal communities displaying the largest differences in diversity and composition. This research has revealed significant shifts in the soil microbiota surrounding remnant kauri fragments, including the loss of microbial taxa with functions in disease suppression and plant health. Kauri dieback disease caused by the soil-borne pathogen *Phytophthora agathidicida* is widespread across remnant kauri forests. This research highlights the need for further assessments into how changes to the soil microbiota surrounding remnant kauri fragments may impact tree health and susceptibility to disease.

3.2 Introduction

The structure and function of forest ecosystems is heavily influenced by their dominant tree species which can shape the characteristics of the surrounding soil and vegetation (Urbanová et al., 2015). Soil microbial communities in forest ecosystems form a vital role in organic matter mineralisation and plant nutrient acquisition, as well as regulating soil biogeochemical processes such as carbon (C) and nitrogen (N) cycling (Zhou et al., 2017). By modifying soil physicochemical properties, plant community composition, litter inputs and chemical composition of root exudates, dominant tree species can influence the diversity and composition of soil microbial communities (Llado et al., 2017; Nakayama et al., 2019). In turn, the tree-associated soil microbiota can influence ecosystem functioning and resilience to disturbance (Liu et al., 2018).

Globally, the conversion and fragmentation of primary forests to plantations and agricultural landscapes is a dominant land use change (Payn et al., 2015). The majority of exotic forestry plantations are found in countries of the Southern Hemisphere where the forestry industry is dominant, such as South America and Oceania (Zhou et al., 2018). In New Zealand, indigenous forest currently covers 6.3 million hectares, whereas planted forest covers 1.73 million hectares. The most common exotic plantation type in New Zealand is of *Pinus radiata* which constitutes approximately 90% of current plantation forestry (MPI, 2018).

Kauri (*Agathis australis*) is one of the most heavily impacted indigenous tree species of New Zealand. Historically, kauri has been subject to heavy disturbance from uncontrolled logging for timber and gum, and then more recently land clearance for pastoral farming and exotic forestry plantations (Ecroyd, 1982; Ogden et al., 2001; Steward & Beveridge, 2010). Belonging to the ancient conifer family *Araucariaceae*, kauri are the largest and longest living tree species in New Zealand with mature tree's having diameters exceeding 3 m, tree heights averaging 30 to 50 m and estimated ages exceeding 1700 years (Steward & Beveridge, 2010). Because of their size, canopy dominance and influences on the biotic and abiotic soil environment, kauri are regarded as ecosystem engineers and are associated with soil podsolization (Wyse et al., 2014). Once covering 1 million hectares, only 7 500 hectares of primary kauri forest remains and exists as fragmented pockets, alongside 60 000 hectares of plantation and secondary regenerating kauri forest (Steward & Beveridge, 2010). The largest remnant primary fragment is located in Waipoua Forest (Northland Region, New Zealand) and is surrounded by pasture and pine plantations (*Pinus radiata*). While kauri forests are essentially unmanaged, pine plantations are subject to intensive management practices such as planting, pruning, thinning, harvest and replanting.

These remnant and regenerating kauri forests are now impacted by kauri dieback disease caused by the soil-borne pathogen *Phytophthora agathidicida*, which is extensive throughout the natural range of kauri and particularly in Waipoua Forest (Beever et al., 2009; Weir et al., 2015). The combined impacts of dieback and widespread forest fragmentation threaten the survival of the remaining kauri forests. Understanding how separate, overlapping disturbance events can interact to influence tree health is of growing interest in forest pathology (Cobb & Metz, 2017). By creating new transmission pathways and altering the environmental conditions of the ecosystem, vegetation and land use changes can impact disease emergence and spread (Meentemeyer et al., 2008). Kauri dieback is an example of widespread tree disease caused by an assumed invasive pathogen that is spreading across a fragmented forest environment, with high levels of spatiotemporal variability at a landscape level. Characterising the changes that have occurred to the soil microbiota as a result of kauri forest fragmentation and plantation establishment is a key step in assessing the impact of historical landscape disturbances on kauri forest health. Additionally, the risks posed by the unintentional introduction of non-native microbial species from plantation establishment to the remnant kauri forests are still unknown (Desprez-Loustau et al., 2007). To facilitate such assessments, the differences between the soil microbial communities associated with kauri and pine forests need to be identified.

The disease triangle is a widely used tool that models disease spread resulting from complex interactions between the pathogen, host and environment (Cobb & Metz, 2017). As kauri forests have been so heavily impacted by forest fragmentation, defining environmental factors conducive to pathogen spread requires us to consider differences in the soil biotic environment surrounding remnant kauri fragments. This study aimed to characterise differences in the diversity and composition of soil fungal and bacterial communities in adjacent stands of kauri and pine plantation forest. Lewis et al. (2019) found that *P. agathidicida* cultures incubated in soils from pine forests produced higher oospore counts *in vitro* than when incubated in soils from kauri forests, where both soils originated from Waipoua Forest. Although no pine plantation soils from Waipoua Forest have yet tested positive for the presence of *P. agathidicida*, the findings of Lewis et al. (2019) suggest the potential for pine forest soils to support survival of *P. agathidicida* and function as a pathogen reservoir. This study seeks to further explore the impact of pine plantation establishment on kauri forest health, by identifying if there have been significant alterations to resident soil microbial communities surrounding remnant kauri forests.

3.3 Methods

3.3.1 Site description and soil sampling

Waipoua Forest (Northland Region, New Zealand) was selected for soil sampling because it contains adjacent stands of old growth kauri forest and pine plantations. A preliminary survey was conducted across Waipoua Forest to identify kauri trees with nearby sites of pine plantation. Once suitable sites were identified, 15 mature kauri trees (not displaying symptoms of dieback) and 15 pine trees from adjacent plantations were sampled. The locations of the sampling sites can be seen in Figure A.1, Appendix A. Pine trees were sampled from third rotation plantations with tree ages of over 50 years. As the pine forest stands have had more active management and have been selectively bred, tree ages and tree structures were uniform and there were few other plant species in each sampling site. For the kauri sites, only mature stands of primary kauri trees over 200 years old were selected for sampling.

For soil sampling, a 500 g composite soil sample that was formed of four 125 g subsamples was collected from around the base of each tree. Following the removal of the leaf litter layer, the A horizon (or the first 10 cm of soil depending on the depth of the A horizon) was targeted for sampling. Each soil sample was sieved, stored in triplicate packaging and transported at 4°C. For each sample, 10 g subsamples were stored at −20°C for DNA extraction. Soils used for chemical analysis were air-dried and passed through a 2 mm sieve to remove leaf litter and debris. Care was taken to prevent the cross-contamination of soil samples by sterilising all sampling equipment between each tree sampled.

The impacts of dieback disease on the soil microbiota associated with kauri are not yet known, therefore, to compare the soil microbial communities of kauri and pine soils it was important to sample soils from kauri not infected with *P. agathidicida*. All kauri and pine soil samples were tested for the presence of *P. agathidicida* using a real-time PCR assay (McDougal et al., 2014; Than et al., 2013) and a soil baiting bioassay (Beever et al., 2010). No kauri or pine soil samples tested positive for *P. agathidicida*. A full description of these methods is provided in Appendix B (Supplementary Methods B.1) and the results are provided in Appendix A (Table A.4).

3.3.2 Soil DNA extraction and amplicon sequencing

Soil DNA was extracted using a DNeasy Powersoil Kit (Qiagen, Germany) following the manufacturer's instructions. Three extractions, using 0.25 g of soil per extraction, were performed on each soil sample and combined to provide a 300 µl DNA extract. DNA samples were quantified

using a Quant-iT dsDNA Assay kit (Invitrogen, California USA) on a Qubit 4 Fluorometer (Invitrogen, California USA). Sample purity was assessed using a Nanodrop Spectrophotometer to check for A260/A280 ratio of over 1.8.

DNA samples were sent to Novogene Co., Ltd (Wan Chai, Hong Kong) for library preparation. The bacterial 16S rRNA gene region was amplified using primers 341F (CCTAYGGGRBGCASCAG) and 806R (GGACTACNNGGGTATCTAAT) to target the V3–V4 gene region (Fadrosh et al., 2014). The fungal internal transcribed spacer (ITS) gene region was amplified using primers ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC) to target the ITS2 gene region (Yang et al., 2018). Following quality control checks, successful libraries were sequenced on the Illumina HiSeq platform to generate 250-bp paired-end reads. Following sequencing, raw sequencing reads were assigned to samples based on unique barcodes attached during library preparation. Barcodes and primers were truncated, and paired-end reads merged using FLASH V1.2.7. These reads were quality filtered using QIIME V1.7.0 (Caporaso et al., 2010) and chimeric sequences were removed using UCHIME.

Quality filtered sequencing reads were clustered into operational taxonomic units (OTUs) based on 97% sequence similarity using UCLUST (Qiime V1.7.0) (Kuczynski et al., 2011). Each OTU was identified taxonomically using the reference databases 'Ribosomal Database Project' for 16S classification and 'UNITE' for ITS classification. OTU clusters were used to create an OTU table showing the abundance of each identified taxa across all the samples. Prior to conducting diversity analyses, OTU tables were filtered to remove non-bacterial and non-fungal taxa. The number of sequencing 16S rRNA and ITS2 sequencing reads in each soil sample can be seen in Table A.3, Appendix A. Due to differences in sequencing depth across samples, fungal OTU tables were rarefied to 30 000 counts per sample and bacterial OTU tables were rarefied to 25 000 counts per sample.

3.3.3 Microbial diversity analysis

The following metrics were selected to assess the bacterial and fungal alpha diversity of kauri and pine soils: observed species counts, Chao1 (an abundance based estimator of species richness), Simpson's diversity (the total species number of a community and the relative abundances of different species making up this community) and Dominance index (how equally taxa are presented across each dataset) (Caporaso et al., 2010). Beta diversity analyses were performed using QIIME diversity plugin scripts and the VEGAN R package (Caporaso et al., 2010; Oksanen et al., 2019). To compare differences in community composition across sites, principal co-ordinates analysis (PCoA) was performed on Bray-Curtis dissimilarity matrices calculated on fungal and bacterial OTU tables.

Differences in dissimilarity scores between kauri and pine sites were tested for significance using Permutational Analysis of Variance (PERMANOVA). Homogeneity of Dispersion (PERMDISP) was used to test for multivariate homogeneity of within group variance. Similarity percentage (SIMPER) analysis was used to identify the individual fungal and bacterial taxa contributing to differences in the Bray–Curtis dissimilarity scores between kauri and pine soils. Euclidean distances were calculated for seven soil properties: pH, total carbon (C), total nitrogen (N), C: N ratio, organic matter, bioavailable N (BN) and BN: N ratio. The pairwise dissimilarities of soil chemical properties were correlated to Bray–Curtis dissimilarities of microbial community composition using Mantel tests, with significance tested through permutations. BIOENV analysis was applied to distance matrices to rank correlate environmental variables with differences in community composition.

The Metacoder R package (Foster et al., 2017) was used to produce heat trees to visualise taxa that had significantly different relative abundances in kauri and pine soils. Differences in the relative abundances of taxa were tested for significance using Wilcoxon rank-sum tests with false discovery rate (FDR) correction. The FUNGuild database (Nguyen et al., 2016) was used to parse fungal OTUs into different ecological categories based on their taxonomic identity.

3.3.4 Soil chemical analyses

Soil samples were sent to Hills Laboratories (Christchurch, New Zealand) where they were air dried at 35°C and sieved < 2mm prior to determination of pH, total carbon (C), organic matter, total nitrogen (N), bioavailable N, and bioavailable phosphorus (P – Olsen P) using established methods (Hinds & Lowe, 1980; Keeney & Bremner, 1966; Nelson & Sommers, 1983; Olsen, 1954).

3.4 Results

3.4.1 Soil microbial diversity

As shown in Table 3.1, there were significant differences in several of the alpha diversity indices between kauri and pine soils. For fungal communities, pine soil had a significantly higher Simpson's diversity than kauri soil. In contrast, kauri soil had a significantly higher dominance, with a few fungal taxa having a large relative abundance in the dataset. For bacterial communities, there were no significant differences between kauri and pine soils for any of the alpha diversity metrics measured. Both kauri and pine soils had a high Simpson's diversity and a low dominance of individual OTUs.

Table 3.1. The mean ± standard error alpha diversity values calculated for fungal and bacterial communities in kauri and pine soils. Results of Wilcoxon rank-sum tests are shown which were used to test for significant differences between kauri and pine soils.

	Diversity index	Kauri	Pine	Significance
Fungi	**Observed species**	982.13 ± 64.88	907.73 ± 81.79	W = 128, p-value > 0.05
	Species richness	1300.6 ± 73.47	1158.8 ± 93.52	W = 121, p-value > 0.05
	Simpson's diversity	0.86 ± 0.03	0.94 ± 0.01	W = 32, p-value < 0.01
	Dominance	0.15 ± 0.02	0.07 ± 0.01	W = 166, p-value < 0.01
Bacteria	**Observed species**	3476.6 ± 68.89	3313.7 ± 142.14	W = 121, p-value > 0.05
	Species richness	4545.8 ± 81.47	4314.6 ± 76.89	W = 136, p-value > 0.05
	Simpsons diversity	0.98 ± 0.00	0.98 ± 0.00	W = 71.5, p-value > 0.05
	Dominance	0.02 ± 0.0	0.01 ± 0.0	W = 129, p-value > 0.05

3.4.2 Soil microbial community composition

There were significant differences in the composition of fungal communities between kauri and pine soils (PERMANOVA pseudo-F = 15.4, p-value = 0.001). Moreover, there were no significant differences in the within group homogeneity of kauri and pine soils (PERMDISP F-value = 0.03, p-value = 0.85), indicating strong and clear differences in the community composition between kauri and pine soils (Figure 3.1). There were significant differences in the composition of bacterial communities between kauri and pine soils (PERMANOVA pseudo-F = 3.9, p-value < 0.001). However, there were also significant differences in the within group homogeneity of fungal and bacterial communities (PERMDISP F-value = 7, p-value < 0.001) which indicates that both group effect and heterogeneous dispersion of samples were having an impact on community dissimilarities (Figure 3.1).

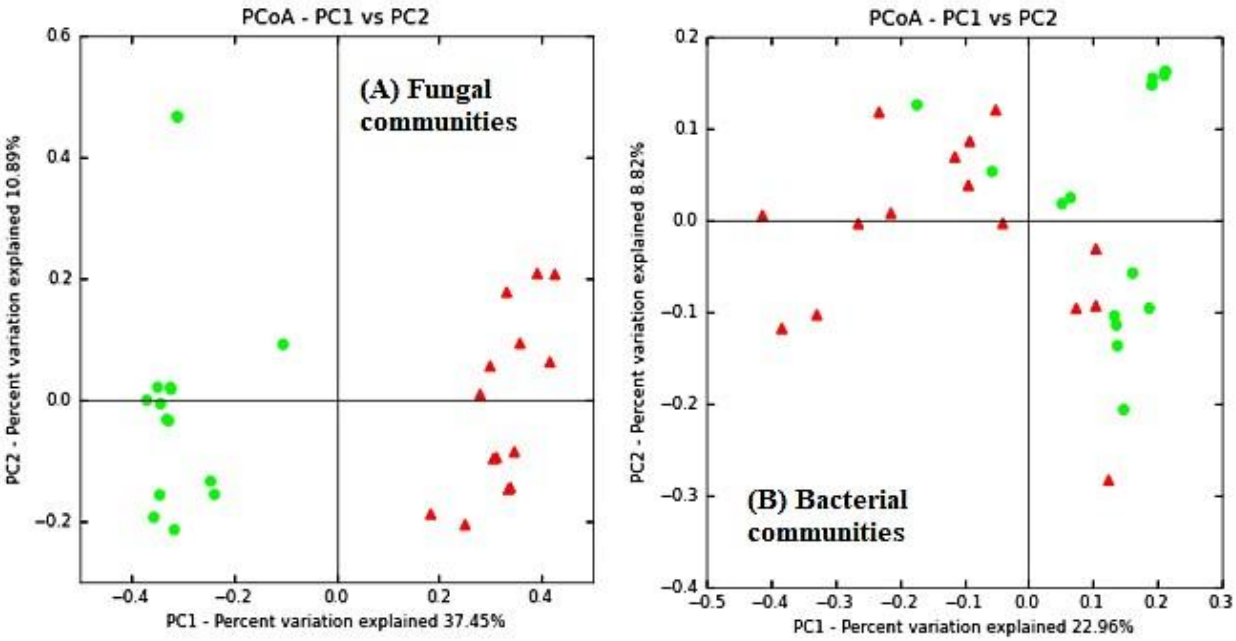

Figure 3.1. Ordination plots of the principal co-ordinates analysis (PCoA) performed using Bray–Curtis dissimilarity matrices of fungal community composition (A) and bacterial community composition (B). Red points represent kauri soil samples and green points represent pine soil samples.

Fungal taxa that significantly contributed to community dissimilarity and were higher in kauri soils included *Apiotrichum gamsii* (11.10% contribution), Tremellomycetes (12.01%), *Trichoderma spirale* (2.45%), Pezizomycotina (2.69%) and Mortierellaceae (1.10%). The fungal taxa with the highest contributions to community dissimilarity that were found higher in pine soils included *Sistotrema* (8.18%), *Oidiodendron chlamydosporicum* (6.32%), *Sagenomella verticillata* (4.22%), *Pseudotomentella griseopergamacea* (3.71%), *Umbelopsis ramanniana* (2.66%), *Oidiodendron*

tenuissimum (2.27%), *Amanita muscaria* (1.85%), Hyaloscyphaceae (3.10%), *Penicillium* (2.38%) and Hypocreales (2.06%). The full results of fungal SIMPER analysis can be found in Table A.1, Appendix A.

Bacterial taxa that significantly contributed to community dissimilarity and were higher in kauri soils included Enterobacteriaceae (8.49% contribution), *Pseudomonas* (1.60%), *Yersinia* (1.23%), Pseudomonadaceae (1.02%), *Acinetobacter* (0.98%) and Rhizobiales (0.78%). Those significantly higher in pine soils included Actinomycetales (4.64%), Acidobacteriaceae (3.17%), Acetobacteraceae (2.29%) and Sinobacteraceae (1.78%). The full results of bacterial SIMPER analysis can be found in Table A.2, Appendix A.

3.4.3 Relationship between community composition and soil physiochemical variables.

There were significant differences found between kauri and pine soils for all soil chemical properties except for pH and Olsen P (Table 3.2). Total C, total N, organic matter, bioavailable N and bioavailable N: total N were significantly higher in kauri soils whilst C: N ratio was significantly higher in pine soils.

Table 3.2. The mean ± standard error values of the soil chemical properties measured for kauri and pine soils. The results of one way ANOVA tests used to test for significant differences in soil chemical properties between kauri and pine soils are shown.

Soil chemical property	Kauri	Pine	Significance
pH	5.16 ± 0.05	5.09 ± 0.10	F-value = 0.37, p-value > 0.05
Olsen P	5.75 ± 0.41	5.5 ± 0.43	F-value = 0.13, p-value > 0.05
Bioavailable N (ug/g)	211.49 ± 7.14	76.13 ± 2.08	F-value = 351.4, p-value < 0.001
Organic Matter (%)	26.18 ± 1.02	18.43 ± 0.29	F-value = 56.49, p-value < 0.001
Total carbon (%)	14.10 ±0.74	10.84 ± 0.13	F-value = 20.08, p-value < 0.001
Total nitrogen (%)	0.57 ± 0.04	0.31 ± 0.01	F-value = 43.40, p-value < 0.001
C:N ratio	27.14 ± 0.24	34.88 ± 1.10	F-value = 43.32, p-value < 0.001
Bioavailable N:Total N	4.01 ± 0.15	2.57 ± 0.16	F-value = 40.04, p-value < 0.001

Mantel tests revealed that bioavailable N (r = 0.72, p-value = 0.001), C: N ratio (r = 0.42, p-value = 0.001), organic matter (r = 0.47, p-value = 0.001), total carbon (r = 0.24, p-value = 0.001) and total nitrogen (r = 0.38, p-value = 0.001) significantly correlated to differences in fungal community composition between kauri and pine soils. Bioavailable N (BIOENV, Spearman's rank = 0.75) was

ranked the best-fitting soil property that accounted for differences in microbial community composition between kauri and pine soils. For bacterial communities, organic matter (r = 0.44, p-value = 0.001), bioavailable N (r = 0.29, p-value = 0.001) and total carbon (r = 0.27, p-value = 0.02) significantly correlated to differences in community composition. Organic matter content was ranked as the best-fitting soil parameter for differences in bacterial community composition between kauri and pine soils (BIOENV, Spearman's rank = 0.42).

3.4.4 Taxonomic composition of microbial communities in kauri and pine soils.

In total, there were 18 fungal phyla identified in kauri and pine soils, with unassigned phyla accounting for 4.39 ± 0.89% of reads in kauri soil and 3.12 ± 0.59% of reads in pine soil. Ascomycota and Basidiomycota formed the majority of reads across all soil samples (Figure 3.2). The relative abundance (%) of Ascomycota (kauri: 38.50 ± 4.20, pine: 56.72 ± 2.46) was significantly higher in pine soil than kauri soil (H-value = 7.92, p-value = 0.005). In contrast, the relative abundance (%) of Basidiomycota (kauri: 49.22 ± 5.39, pine: 31.58 ± 2.46) was significantly higher in kauri soil than pine soil (H-value = 4.98, p-value = 0.026).

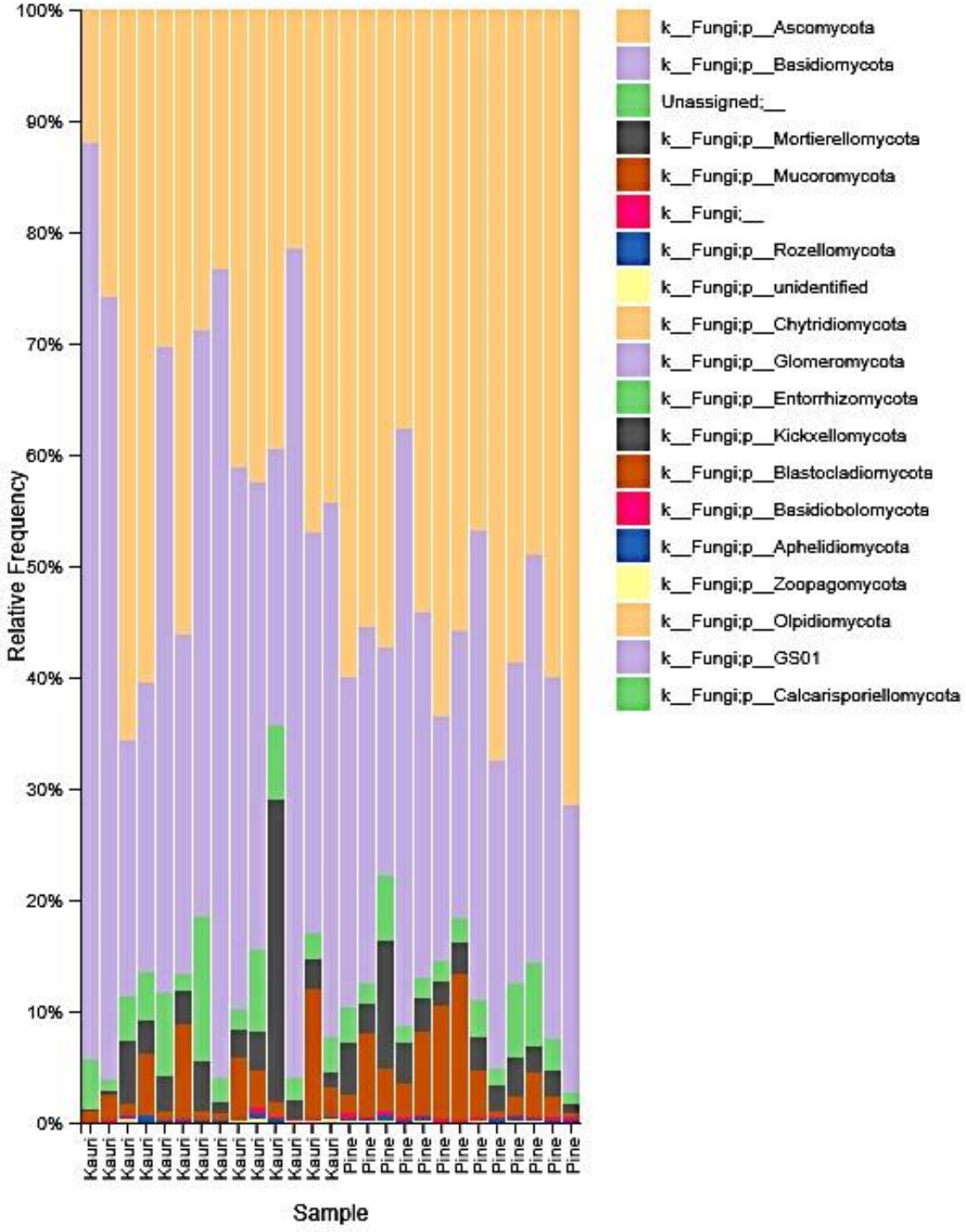

Figure 3.2. The relative abundance (%) of fungal phyla in all kauri and pine soil samples analysed.

Several fungal taxa were found to have significant differences in their relative abundances between kauri and pine soils (Figure 3.3). Within the Basidiomycota, the class Tremellomycetes was significantly higher in kauri soil (p-value < 0.001), including the Trichosporonales (p-value < 0.001), Trichosporonaceae (p-value < 0.001) and *Apiotrichum* (p-value < 0.001). Members of the Agaricales had significantly higher relative abundance in kauri soil, such as *Clavulinopsis* (p-value = 0.015) and

Hygrocybe (p-value = 0.042). The family Ganodermataceae (p-value = 0.015) its associated genus *Ganoderma* (p-value = 0.015) also had a significantly higher relative abundance in kauri soil. Two families belonging to the Mucorales, Backusellaceae (p-value = 0.015) and Mucoraceae (p-value = 0.004), had a significantly higher relative abundance in kauri soil. This included their genera *Backusella* (p-value = 0.015) and *Mucor* (p-value = 0.004). Members of the Ascomycota with significantly higher relative abundance in kauri soil included the Saccharomycetaceae (p-value = 0.029), *Barnettozyma* (p-value = 0.010) and *Kazachstania* (p-value = 0.029). The Hypocreales (p-value = 0.019), Hypocreaceae (p-value = 0.003) and *Trichoderma* (p-value = 0.003) were also in significantly higher relative abundance in kauri soil.

For pine soil, members of the Basidiomycota found in significantly higher relative abundance included the Agaricomycetes (p-value = 0.008), *Thelophorales* (p-value < 0.001), Cantharellales (p-value < 0.001) and Atheliales (p-value < 0.001). This included the genera *Sistrotrema* (p-value < 0.001), *Amanita* (p-value = 0.002), *Inocybe* (p-value = 0.025), *Tomentella* (p-value < 0.001), *Pseudomentella* (p-value < 0.001), *Tylospora* (p-value < 0.001) and *Rhizopogon* (p-value < 0.001). Additionally, the orders Tremellales (p-value < 0.001) and Leucosporidiales (p-value < 0.001) had significantly higher relative abundance in pine soil. Members of the Ascomycota with significantly higher relative abundance in pine soil included the families Ophiocordycipotaceae (p-value = 0.008) and Trichocomaceae (p-value < 0.001) and the genera *Beauveria* (p-value = 0.029), *Aspergillus* (p-value < 0.001), *Sagenomella* (p-value < 0.001), *Talaromyces* (p-value = 0.005), *Cladophialophora* (p-value = 0.002) and Venuriaceae (p-value = 0.010). The Leotiomycetes were also significantly higher in pine soil, which included the Helotiales (p-value < 0.001) and the genera *Oidiodendron* (p-value < 0.001), *Meliniomyces* (p-value = 0.015), *Scytalidium* (p-value = 0.020) and *Phialocephala* (p-value < 0.001). The Umbelopsidomycetes (p-value = 0.008) were significantly higher in pine soil, including its order Umbelopsidales (p-value = 0.008), family Umbelopsidaceae (p-value = 0.008) and genus *Umbelopsis* (p-value = 0.008).

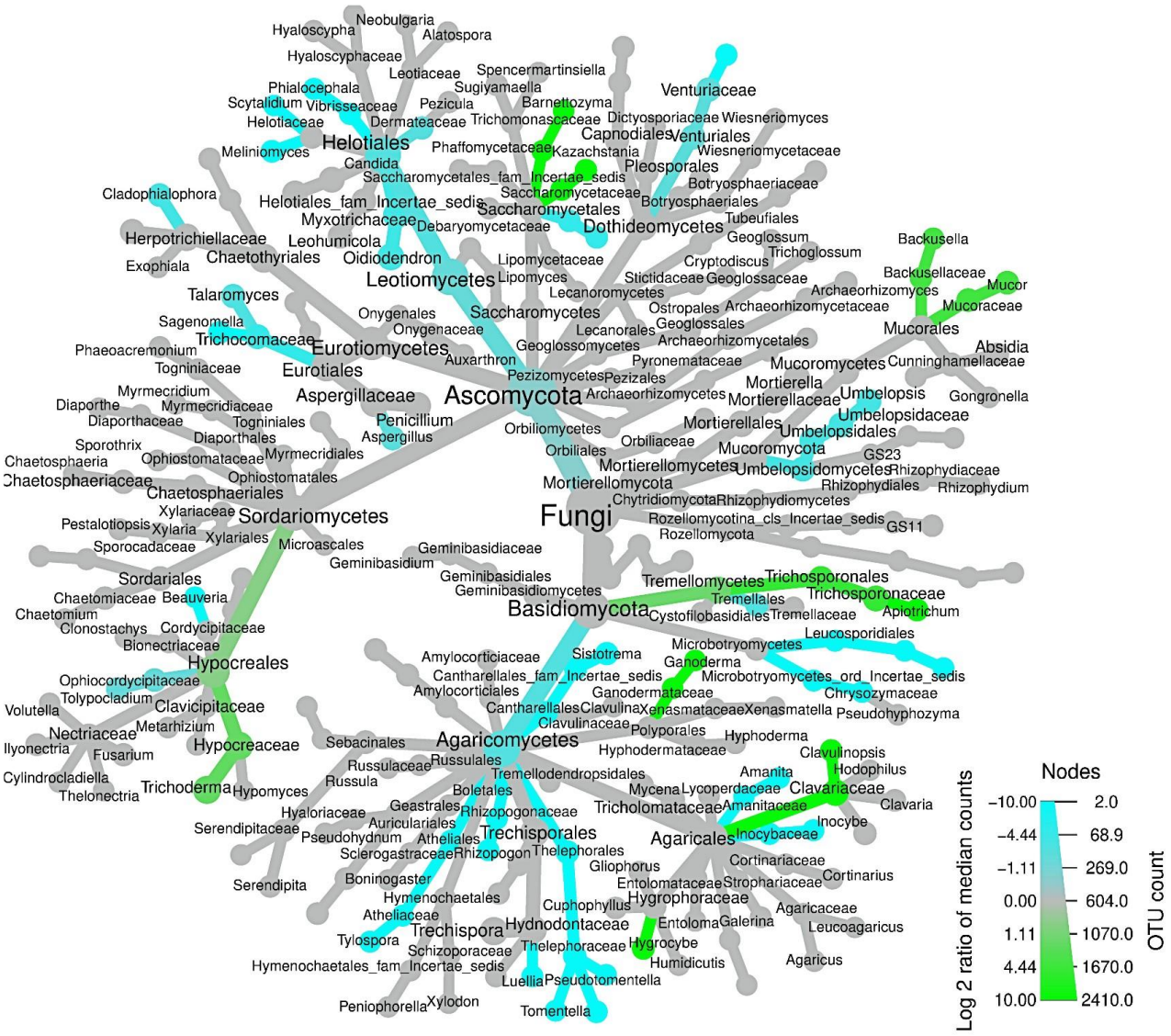

Figure 3.3. The fungal taxa which had significantly different relative abundances (p-value < 0.05) in kauri and pine soils. Fungal taxa that were significantly higher in kauri soil are represented by green nodes and those that were significantly higher in pine soil are represented by blue nodes.

Although there were 51 bacterial phyla identified in kauri and pine soils, a large majority of these had low relative abundances and only the 20 most abundant phyla are shown in Figure 3.4. Three bacterial phyla dominated kauri and pine soils which were the Proteobacteria (kauri %: 56.33 ± 2.61, pine %: 49.10 ± 1.38), Actinobacteria (kauri %: 18.95 ± 1.4, pine %: 23.07 ± 1.24) and Acidobacteria (kauri %:12.91 ± 0.95, pine %: 15.79 ± 0.93). Proteobacteria had a significantly higher relative abundance (%) in kauri soils (H-value = 4.39, p-value = 0.036), whereas Actinobacteria (H-value = 4.29, p-value = 0.026) and Acidobacteria (H-value = 4.05, p-value = 0.044) had a significantly higher relative abundance (%) in pine soils.

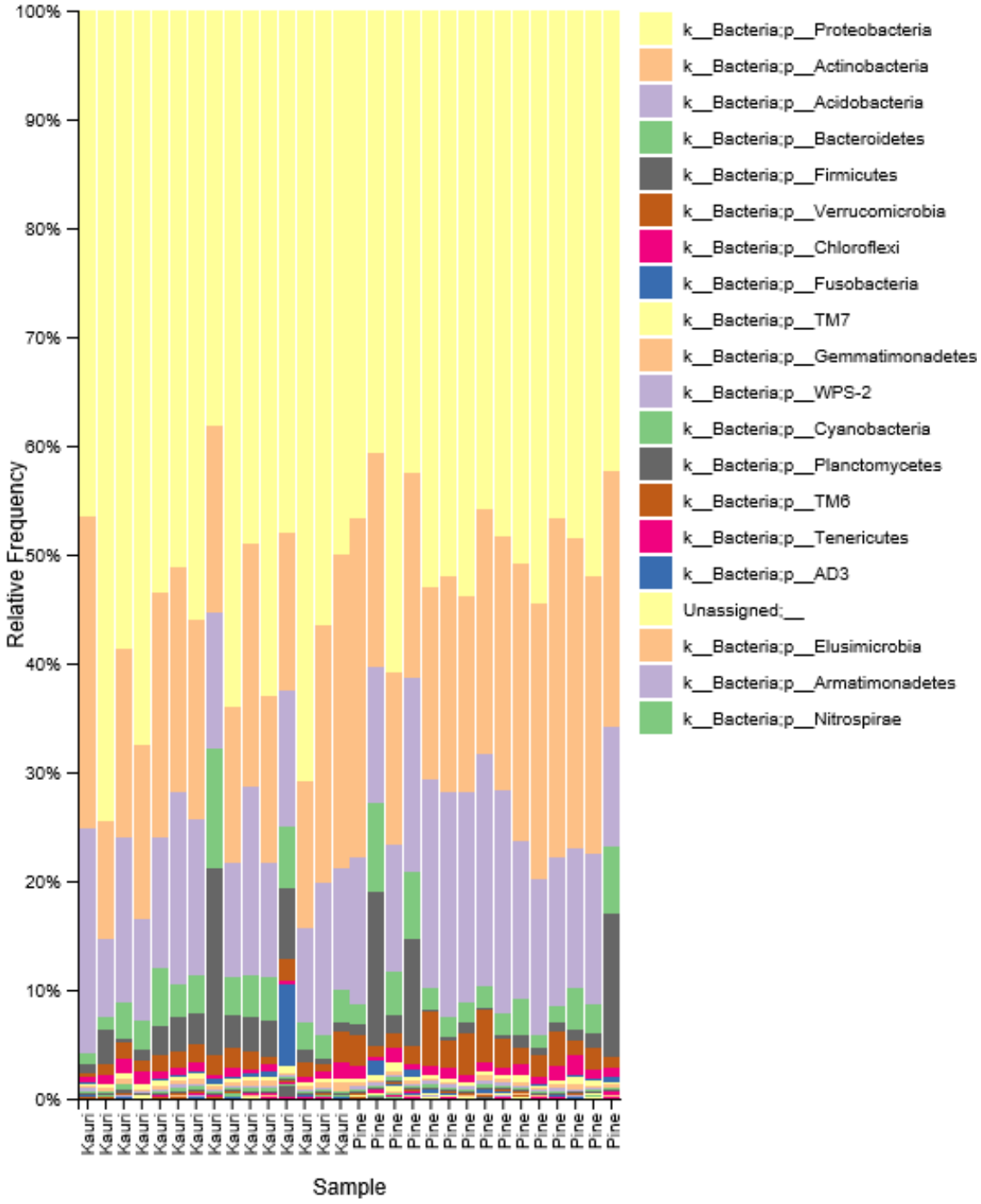

Figure 3.4. The relative abundances (%) of bacterial phyla in all kauri and pine soil samples analysed.

In contrast to fungal communities, fewer bacterial taxa had significant differences in their relative abundance between kauri and pine soils (Figure 3.5). The orders Neisseriales (p-value = 0.048), Pseudomonadales (p-value = 0.006), Enterobacteriales (p-value = 0.006) and Rhodobacterales (p-value = 0.016) had significantly higher relative abundances in kauri soil. This included the families Neisseriaceae (p-value = 0.048), Pseudomonadaceae (p-value = 0.008), Enterobacteriaceae (p-value

= 0.006), Rhizobiaceae (p-value = 0.022), Rhodobiaceae (p-value = 0.022) and Moraxellaceae (p-value = 0.026). Members of the Proteobacteria found to be significantly higher in kauri soil included *Pseudomonas* (p-value = 0.008), *Acinetobacter* (p-value = 0.022), *Sphingomonas* (p-value = 0.024), *Afifiella* (p-value = 0.022) and *Phenylobacterium* (p-value = 0.022). One family belonging to Actinobacteria, Micromonosporaceae (p-value = 0.022), was significantly higher in kauri soil. Three families belonging to the Proteobacteria, Alcaligenaceae (p-value = 0.027), Burkholderiaceae (p-value = 0.032) and Acetobacteraceae (p-value = 0.006), had a significantly higher relative abundance in pine soil, which included the genera *Burkholderia* (p-value = 0.022) and *Acidocella* (p-value = 0.006). One family belonging to the phylum Acidobacteria, Koribacteraceae (p-value = 0.020), was in significantly higher relative abundance in pine soil.

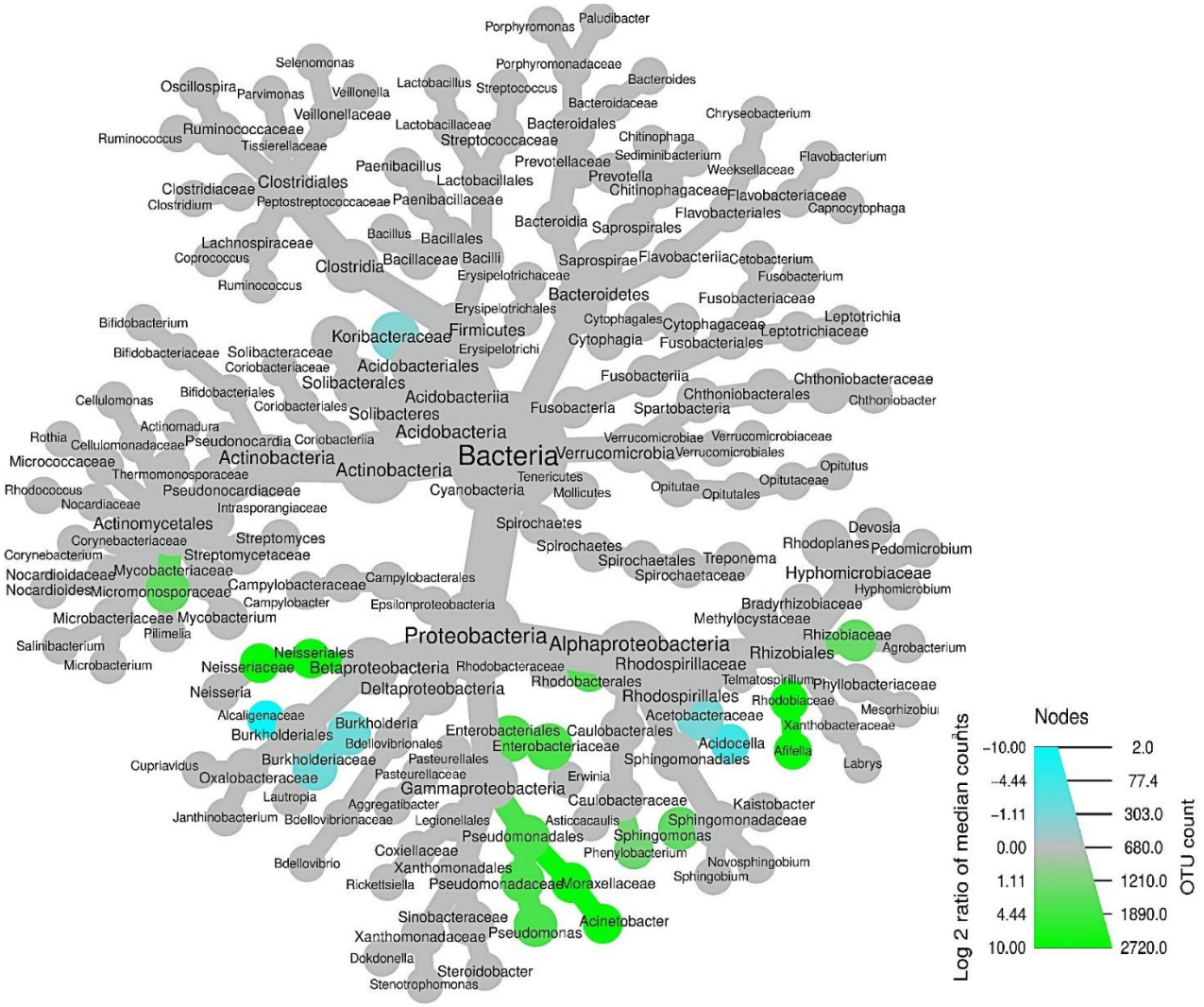

Figure 3.5. The bacterial taxa which had significantly different relative abundances (p-value < 0.05) in kauri and pine soils. Bacterial taxa that were significantly higher in kauri soil are represented by green nodes and those that were significantly higher in pine soil are represented by blue nodes.

3.4.5 FUNGuild analysis

The results of FUNGuild analysis performed on fungal communities of kauri and pine soils are presented in Table 3.3. When looking at the differences in trophic modes of fungal communities between kauri and pine soils, kauri soil had a significantly higher relative abundance of saprotrophic fungi whereas pine soil had a significantly higher relative abundance of symbiotrophic fungi. When looking at ecological guilds, pine soil had a significantly higher relative abundance of ectomycorrhizal fungi and undefined saprophytes. In contrast, kauri soil had a significantly higher relative abundance of soil saprophytes, endophytes and ericoid mycorrhizae. In relation to fungal growth forms, kauri soil had a significantly higher abundance of yeasts whereas pine soil had a significantly higher abundance of dark septate endophytes, agaricoid fungi and macrofungi.

Table 3.3. The mean ± standard error relative abundance (%) of each FUNGuild category in kauri and pine soils. The results of Kruskal–Wallis chi-squared tests are displayed which were used to test for significant differences in the relative abundance (%) of each FUNGuild category between kauri and pine soils.

FUNGuild category		Kauri %	Pine %	Significance
Trophic mode	Saprotroph	70.07 ± 3.59	48.33 ± 3.62	H-value = 10.43, p-value < 0.001
	Pathotroph	2.19 ± 0.38	2.06 ± 0.24	H-value = 0.19, p-value > 0.05
	Symbiotroph	13.53 ± 2.13	28.47 ± 0.84	H-value = 15.77, p-value < 0.001
Ecological guild	Soil saprophyte	45.41 ± 5.01	6.82 ± 1.39	H-value = 20.60, p-value < 0.01
	Litter saprophyte	0.65 ± 0.40	0.34 ± 0.10	H-value = 2.33, p-value > 0.05
	Undefined saprophyte	18.76 ± 2.42	36.84 ± 4.0	H-value = 11.00, p-value < 0.001
	Wood saprophyte	4.58 ± 2.3	2.46 ± 0.72	H-value = 0.76, p-value > 0.05
	Plant pathogen	1.33 ± 0.30	1.62 ± 0.18	H-value = 3.20, p-value > 0.05
	Ectomycorrhizae	0.41 ± 0.08	11.98 ± 1.33	H-value = 21.00, p-value < 0.001
	Endophyte	12.02 ± 2.03	6.46 ± 0.73	H-value = 6.19, p-value < 0.05
	Ericoid mycorrhizae	17.64 ± 0.39	12.01 ± 1.57	H-value = 11.29, p-value < 0.001
Growth form	Agaricoid	2.44 ± 1.33	6.41 ± 1.20	H-value = 12.81, p-value < 0.001
	Microfungus	17.28 ± 2.88	22.32 ± 3.92	H-value = 1.95, p-value > 0.05
	Macrofungus	5.05 ± 2.34	10.79 ± 1.76	H-value = 8.05, p-value < 0.01
	Yeast	43.62 ± 5.00	3.93 ± 0.48	H-value = 21.00, p-value > 0.001
	Dark septate endophyte	1.15 ± 0.32	11.75 ± 1.61	H-value = 10.71, p-value < 0.001

3.5 Discussion

3.5.1 Differences in microbial diversity and composition between kauri and pine soils.

When comparing the soil microbial communities between kauri and pine soils, fungal communities displayed the most significant differences in diversity and composition. Although there were significant differences in bacterial communities between kauri and pine soils, differences in forest soil type appear to be exerting a greater influence on fungal communities.

Pine soil had a significantly higher fungal diversity compared to kauri soil, with the fungal communities of kauri soils dominated by a few taxonomic groups with large relative abundances. This is consistent with previous studies that have observed soils from plantation forests to have a higher fungal diversity than primary forests (Zhou et al., 2018). The high dominance of a few fungal taxa in kauri soil may be a result of the selective soil environment of kauri forests, which over time can act as an ecological filter and only supports well-adapted taxonomic groups. The influence and selection pressure that kauri exerts on its surrounding environment has already been observed for plant communities (Wyse et al., 2014). Typically, ecosystems with a lower resident diversity are more vulnerable to invasion as they have a greater empty niche space and resource availability (Bonanomi et al., 2014; van Elsas et al., 2012). This finding may pose an interesting question as to whether the relatively low fungal diversity of kauri soils (McKenzie et al., 2002) could predispose them to pathogen invasion. Bacterial communities of both kauri and pine soils had a high taxonomic diversity and were dominated by the phyla Proteobacteria, Actinobacteria and Acidobacteria which are all commonly found in acidic coniferous forests (Llado et al., 2017). The non-significant differences in bacterial diversity between kauri and pine soils may be explained by their non-significant differences in soil pH, which is often regarded as a strong predictor of bacterial community composition (Lauber et al., 2009; Llado et al., 2017).

The clear differences in fungal community composition between kauri and pine soils may be explained ecologically, as the saprophytic and symbiotic roles of soil fungi means their composition is often related to tree litter inputs and root exudates (Urbanová et al., 2015). In fact, fungal biomass and community structure are regarded as more credible indicators of environmental change than organic matter status (Wu et al., 2019). In contrast, changes to dominant tree species produce smaller effects on soil bacterial communities as they occupy niche space on a smaller scale and are less directly influenced by the activities tree roots (Urbanová et al., 2015). The higher organic matter content of kauri soil could explain its dominance of saprotrophic fungi which form a key role in organic matter decomposition (Wu et al., 2019). Saprophytic fungi have been observed to be more

abundant in high nutrient soils, such as kauri soils. In contrast, low nutrient soils are typically more diverse in ectomycorrhizal fungi as observed in this study for pine soils (Wu et al., 2019). Bioavailable nitrogen, which can be an indicator of soil quality and microbial biomass (Wakelin et al., 2013), was strongly correlated to differences in fungal community composition between kauri and pine soils. The higher values of bioavailable N in kauri soils and its correlation to differences in fungal community composition may indicate that kauri soils have a higher fungal biomass and activity, although more direct measures of fungal activity need to be performed to support this notion.

3.5.2 Potential threats posed by introduced microbial taxa.

Forestry plantation establishment can result in the increased introduction of non-native fungi (Desprez-Loustau et al., 2007), a factor that may be contributing to the higher fungal diversity observed in pine soil in this study. Kauri trees have previously been reported to not associate with ectomycorrhizal (ECM) fungi (McKenzie et al., 2002) and in this study kauri soil had low abundances of ECM fungi, especially when compared to pine soils. The establishment of pine plantations has been observed to result in higher abundances of ECM fungi (Chapela et al., 2001; Giachini et al., 2000) and the ability of *P. radiata* to form mutualistic associations with non-native ECM fungi has been credited with their invasive success across New Zealand (Dickie et al., 2010). Many fungal taxa found in significantly higher relative abundance in pine soils in this study (i.e. *Inocybe, Pseudotomentella, Rhizopogon, Amanita, Thelephora, Phialocephala*, *Tylospora* and Cantharellales) have previously been reported as non-native ECM associates of *P. radiata* in New Zealand (Dickie et al., 2010; Walbert et al., 2010).

New Zealand's kauri forests, which have historically been subject to extensive deforestation and land clearance, are currently threatened by the spread of kauri dieback (Beever et al., 2009; Steward & Beveridge, 2010). Forest ecosystems worldwide are threatened by both anthropogenic-induced environmental disturbances, such as habitat fragmentation, and the more frequent exposure to new diseases from increasing global trade (Thakur et al., 2019). The unintentional introduction of invasive fungi and oomycetes by introduced tree species is a common invasion pathway, with invasive pathogens being a driver of emerging infectious diseases in forest ecosystems (Desprez-Loustau et al., 2007; Ghelardini et al., 2016). This risk is particularly high for *Phytophthora* pathogens, with the plant nursery trade being identified as a vector for their introduction and dispersal into new ecosystems (Jung et al., 2016; Scott et al., 2013). This study found significant differences in the soil microbial communities of native kauri soils compared to exotic pine plantations, these differences could potentially expose kauri fragments to newly introduced microorganisms which now have kauri within their range. The presence of fungal taxa that have previously been reported as non-native to

New Zealand provides early evidence to support this. The potential ecological consequences of non-native fungi that have yet to cause negative ecological impacts are rarely quantified (Desprez-Loustau et al., 2007). Finding methods to quantify the risks associated with the shifts in microbial communities presented in this study would be wise, especially given the current threatened state of the remnant kauri fragments.

3.5.3 Potential role of soil microbial communities to the health of remnant kauri fragments.

Characterising the belowground diversity of native forest ecosystems is required to identify microbial indicators of soil quality that may support long-term tree health (Schloter et al., 2018). This study identified that microbial taxa such as *Apiotrichum, Tremellomycetes, Trichoderma, Pseudomonas, Enterobacteriaceae, Actineobacter* and *Rhizobiaceae* were found in significantly higher relative abundance in kauri soil. Several of these genera, such as *Pseudomonas* and *Trichoderma*, are known for their roles in plant disease suppression and promotion of plant health (Avis et al., 2008). The *Rhizobiaceae* family contains many nitrogen-fixing endophytes with key roles in plant growth promotion (Gaiero et al., 2013). Additionally, the *Enterobacteriaceae* have been identified as plant growth-promoting bacteria with roles in aiding plant nitrogen and phosphorus uptake and facilitating glucose decomposition in soils (Degelmann et al., 2009; Kumar et al., 2017). The loss of core members of the microbiota from soils surrounding remnant kauri fragments could alter the forest's ability to respond to pathogen invasion, particularly if these soil microorganisms have potential roles in soil and plant health. The findings of this study support the hypothesis that the establishment of pine plantations has altered the soil microbiota across Waipoua Forest. Understanding the ecological impacts of these changes to the soil microbiota surrounding remnant kauri forests is important to protect the long-term health and functioning of fragmented kauri forests.

Pathogen spill over occurs when pathogens have multiple hosts across a landscape. When these alternative hosts are able to support higher pathogen inoculum loads, disease transmission can be accelerated across the environment (Beckstead et al., 2010). It is well understood that *P. agathidicida* is highly pathogenic against kauri and forms an extensive host-pathosystem across remnant kauri forests. A previous study by Lewis et al. (2019) identified that the oospore counts of *P. agathidicida* were significantly higher when cultures were incubated in pine soils compared to kauri soils. Despite the findings of these *in vitro* studies, no environmental pine soil samples have yet tested positive for *P. agathidicida* either in this study or by Lewis et al. (2019). Furthermore, the fungal diversity of pine soils was shown to be significantly higher than kauri soils. Given that high

fungal diversity has previously shown a negative relationship with pathogen invasion (Bonanomi et al., 2014), this goes against the proposal that pine soils might function as a pathogen reservoir. However, results from this research show that there are significant differences in the soil microbial communities of kauri and pine soils. Further work is needed to determine how these differences in soil microbial diversity and structure may influence the growth and behaviour of *P. agathidicida* and health of remnant kauri fragments.

3.6 Conclusion

This research has identified significant differences in the diversity and composition of soil microbial communities associated with old growth kauri forests and adjacent pine plantations in Waipoua Forest. Considering the current threats posed to kauri forests by dieback disease, it is essential for us to further understand of how these differing microbial communities impact the health of kauri forest fragments and their ability to respond to pathogen invasion. This study acts as a stepping stone for facilitating such future research by being the first to fully characterise the fungal and bacterial communities of New Zealand's native kauri forests in comparison with those of exotic pine plantations.

3.7 Acknowledgements

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Chapter 4

Soil microbial community restructuring and functional changes in ancient kauri (*Agathis australis*) forests impacted by the invasive pathogen *Phytophthora agathidicida*.

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Supplementary data for this chapter is provided in Appendix B.

4.1 Abstract

New Zealand's ancient kauri (*Agathis australis*) forests are under threat from the spread of dieback disease caused by the soil-borne pathogen *Phytophthora agathidicida*. Kauri function as a foundation species in their forests by supporting an ecologically distinct plant and soil environment. Therefore, their widespread dieback could cause a series of secondary, cascading impacts that have the potential to impact long-term forest ecosystem functioning. The impacts of disease outbreak and subsequent tree dieback on the soil microbiota of kauri forests remains unknown. Using high throughput 16S rRNA and ITS gene region sequencing and GeoChip 5S microarray analysis, this research analysed the differences in diversity, taxonomic composition and functional genes related to carbon (C) and nitrogen (N) cycling of soil microbial communities associated with asymptomatic and symptomatic mature kauri. Results have found significant differences in the fungal diversity and the fungal and bacterial community composition between asymptomatic and symptomatic kauri. Several microbial taxa previously known for their roles in plant disease suppression, such as *Penicillium, Trichoderma, Enterobacteriaceae* and *Pseudomonas*, were found in significantly higher relative abundance in asymptomatic kauri soils. The findings of this study have provided a promising direction for the discovery of disease suppressive microorganisms against kauri dieback. In addition, significant differences were found in the composition and abundance of microbial genes related to C and N cycling which highlights the potential long term impacts of dieback disease on the health and functioning of kauri forests.

4.2 Introduction

Globally, large-scale forest disease outbreaks are increasing at alarming rates as a result of biological invasions, climate change and anthropogenic disturbances (Cobb & Metz, 2017; Edburg et al., 2012; Steffen et al., 2015). The implications of this are dire, as forests form critical roles in maintaining biodiversity, carbon (C) storage and climate regulation (Davidson & Janssens, 2006; Hui et al., 2017; Nolan et al., 2018). Soil-borne pathogens are known to affect soil microbial communities and their functional responses in highly managed monoculture systems (Dignam et al., 2019; Dignam et al., 2018), including the cycling of C and nitrogen (N). However, little is known about the impact of emerging soil pathogens on old growth, tree dominated forest ecosystems.

Kauri (*Agathis australis*) is one of New Zealand's most ancient native tree species and are currently under threat from the spread of dieback disease caused by *Phytophthora agathidicida* (Beever et al., 2009; Waipara et al., 2009). Kauri are an iconic and culturally significant tree species for New Zealand, being regarded by the indigenous Māori as their living ancestors (Black et al., 2018). They function as a foundation species in their namesake forests, significantly influencing surrounding plant species composition and supporting the most species rich forest type in New Zealand (Ogden, 1995; Wyse et al., 2014). Kauri forests have faced a long history of disturbance and currently less than 1% of primary kauri forest remains (Steward & Beveridge, 2010). Given the significance and threatened status of kauri forests, we urgently seek solutions to effectively manage the spread of dieback and protect the remnant ancient kauri forests.

In the face of pathogen invasion, soil microbial communities can modulate their activity and functional responses to promote plant host health and provide pathogen defence (Desprez-Loustau et al., 2007; Raaijmakers & Mazzola, 2016). For soil-borne pathogens such as *P. agathidicida*, host infection begins at the root zone when motile zoospores encyst at the root surface and colonize the root via penetrative hyphae (Bellgard et al., 2016). Consequently, they must interact with the host's associated soil microbiota throughout the infection event. The diversity, abundance and functional roles of the soil microbiota influence how they interact with invading pathogens and this interaction is a key factor in determining whether the pathogen can successfully spread, establish, and elicit host infection (Kemen, 2014; Thakur et al., 2019). Studying shifts in diversity and composition of the soil microbiota associated with host-pathogen infection events can guide the identification of taxa which can suppress disease development (Penton et al., 2014). However, no studies have yet examined the response of the kauri soil microbiota to the expression of dieback disease. Furthermore, there are only a limited number of studies published on the soil microbiota associated with kauri (McKenzie et al., 2002; Morrison & English, 1967; Padamsee et al., 2016). Therefore, this

study aimed to characterise differences in the diversity and taxonomic composition of soil microbial communities associated with asymptomatic and symptomatic kauri across Waipoua Forest (Northland Region, New Zealand).

P. agathidicida is a highly virulent pathogen of kauri, with all known cases of infection resulting in tree mortality (Horner & Hough, 2014). Aside from initial tree mortality, the cascading effects following loss of a foundation species can impact long term ecosystem functioning and biogeochemical cycling (Avila et al., 2016; Boyd et al., 2013; Edburg et al., 2012; Lovett et al., 2010; Peltzer et al., 2010). For example, large stores of C and immobilised N are a distinctive feature of kauri forest soils (Macinnis-Ng & Schwendenmann, 2015; Wyse et al., 2014) and changes to these properties following kauri dieback could greatly impact long term C and N cycling in kauri forests. Soil microorganisms play key roles in biogeochemical cycling and ecosystem functioning and it is important to quantify the impacts of tree dieback on their functional potential (He et al., 2007). Therefore, this study also aimed to assess the secondary impacts of kauri dieback on soil microbial function by studying the differences in functional genes related to C and N cycling between asymptomatic and symptomatic kauri trees.

4.3 Methods

4.3.1 Soil collection

Soil sampling sites were located within Waipoua Forest (Northland Region, New Zealand) (**Section 1.3**). A preliminary survey (**Section 1.3**) was conducted across Waipoua Forest to confirm there were enough asymptomatic and symptomatic kauri trees present in infected forest stands. Four forest sites containing kauri of a similar size, age (approximately 200 years old), canopy dominance and degree of disease expression were selected for sampling. Soil samples were collected from 20 asymptomatic and 20 symptomatic kauri which were diagnosed based on classic kauri dieback symptomology (Waipara et al., 2013). Symptomatic kauri expressing the later stage symptoms of dieback which included lower trunk gummosis, root and wood rot, extensive leaf loss and chlorosis were selected for sampling. Within each site, the asymptomatic and symptomatic kauri selected for sampling were within a 200 m distance from one another (Figure B.1, Appendix B).

For soil sampling a 500 g composite sample, formed of four 125 g subsamples, was taken from the cardinal points around the base of each tree (Waipara et al., 2013). Following the removal of the litter layer, the A horizon (or top 10cm of soil depending on the depth of the A horizon) was targeted for sampling. Each soil sample was sieved, stored in triple contained biohazard labelled packaging and transported at 4°C. Samples were split into 10 g subsamples stored at -20^{o}C for DNA analysis,

200 g subsamples stored at 4°C for pathogen detection assays and 200 g subsamples stored for chemical analysis.

4.3.2 Pathogen bioassays

A soil baiting bioassay (Bellgard et al., 2013) and real-time PCR assay (McDougal et al., 2014; Than et al., 2013) was used to confirm that symptomatic soil samples tested positive for *P. agathidicida* and asymptomatic soil samples tested negative for *P. agathidicida*. A full description of these methods (Supplementary Methods B.1) and results (Table B.2) are provided in Appendix B.

4.3.3 Soil chemical analyses

Soil samples were sent to Hills Laboratories (Christchurch, New Zealand) where they were air dried at 35°C and sieved < 2mm prior to determination of pH, total carbon (C), organic matter, total nitrogen (N), bioavailable N, and bioavailable phosphorus (P – Olsen P) using established methods (Hinds & Lowe, 1980; Keeney & Bremner, 1966; Nelson & Sommers, 1983; Olsen, 1954).

4.3.4 Soil DNA extraction and amplicon sequencing

Methods for soil DNA extraction and amplicon sequencing followed those previously outlined in **Section 3.3.2**. The following steps were performed using Qiime 2 2019.4 (Bolyen et al., 2019). Sequencing reads were clustered into OTUs at 99% sequence similarity and a representative sequence from each OTU was identified taxonomically using reference databases 'Green Genes 13.8' for 16S rRNA classification and 'UNITE 18.11.2018' for ITS classification. The resulting OTU cluster, with taxonomic assignment, was used to create OTU and taxonomic frequency tables representing the abundance of identified taxa in each sample. Frequency tables were filtered to remove non-bacterial or fungal taxa and low abundance taxa which had less than 10 read counts. Fungal OTU frequency tables were rarefied to 27 500 counts per sample and bacterial OTU frequency tables were rarefied to 30 000 counts per sample. Alpha diversity analyses were performed on rarefied OTU frequency tables using the Qiime 2 'diversity' plugin. The Phyloseq R package (McMurdie & Holmes, 2013) was used to analyse and visualise the OTU frequency and taxonomic datasets. Beta diversity analyses were performed using Bray-Curtis dissimilarity matrices and visualised using non-metric multi-dimensional scaling (NMDS) plots. Unweighted and weighted UniFrac distance matrices were also calculated for bacterial communities to measure for differences in phylogenetic beta diversity between asymptomatic and symptomatic soils. Kruskal Wallis tests were used to identify taxonomic classes and orders which had significantly different relative abundances between asymptomatic and symptomatic kauri soils. DESeq2 analysis was used to identify OTUs which were

found to have significantly different abundances between asymptomatic and symptomatic kauri soil (Love et al., 2014).

4.3.5 GeoChip 5S microassay analysis

GeoChip 5S microarray analysis was selected to explore the functional attributes of soil microbial communities (Shi et al., 2019). Large stores of C and immobilised N are two distinctive properties of kauri forests (Macinnis-Ng & Schwendenmann, 2015; Ogden, 1995) and to keep the microarray analysis focused on the objectives of this study, only genes related to carbon and nitrogen cycling were retained for further analysis. Five DNA extracts of asymptomatic kauri soils and five DNA extracts of symptomatic kauri soils were sent to the Institute for Environmental Genomics (University of Oklahoma, USA) where the microarray analysis was completed. To analyse the functional gene data, alpha diversity calculations were performed using the Institute of Environmental Genomics 'MicroAnalysis' data analysis software. Beta diversity analyses were performed using Bray-Curtis dissimilarity matrices and visualised in PCoA ordination plots using the 'Phyloseq' R package. Differential abundance testing of individual carbon and nitrogen cycling genes between asymptomatic and symptomatic kauri soils was performed using DESeq2 (Love et al., 2014).

4.4 Results

4.4.1 Symptomatic kauri soils have increased fungal diversity and altered community composition

For fungal communities, the number of observed OTUs, Shannon diversity and Pielou's evenness were significantly higher in symptomatic kauri soil than asymptomatic kauri soil (Table 4.1). For bacterial communities, there were no significant differences in observed OTUs, Shannon diversity, Faith's phylogenetic diversity or Pielou's evenness between asymptomatic and symptomatic kauri soils (Table 4.1).

Table 4.1. The mean ± standard error alpha diversity values calculated for fungal and bacterial communities in symptomatic and asymptomatic kauri soils. The results of Kruskal–Wallis chi-squared tests are displayed which were used to test for significant differences in alpha diversity between asymptomatic and symptomatic soils.

	Alpha diversity	Asymptomatic	Symptomatic	Significance
Fungi	**Shannon diversity**	5.38 ± 1.09	7.13 ± 0.97	H-value = 13.47, p-value <0.001
	Pielou's evenness	0.49 ± 0.08	0.59 ± 0.07	H-value = 11.15, p-value <0.001
	Observed OTUs	2103.47 ± 559.41	3799.93 ± 698.13	H-value = 19.15, p-value <0.001
Bacteria	**Shannon diversity**	9.86 ± 0.46	9.89 ± 0.43	H-value = 0.00, p-value = 0.95
	Pielou's evenness	0.79 ± 0.01	0.79 ± 0.01	H-value = 0.12, p-value = 0.72
	Observed OTUs	5567.53 ± 935.56	5675.60 ± 1275.65	H-value = 0.02, p-value = 0.88
	Faith's diversity	55.52 ± 1.52	52.30 ± 1.29	H-value = 2.42, p-value = 0.12

There was a significant difference in the fungal community composition (ANOSIM R = 0.46, p-value = 0.001) and bacterial community composition (ANOSIM R = 0.17, p-value = 0.004) between asymptomatic and symptomatic kauri soils (Figure 4.1). However, the R test statistic score for fungal communities (0.46) was higher than bacterial communities (0.17) suggesting that the differences in composition between asymptomatic and symptomatic kauri soils is stronger for fungal communities than bacterial communities. There was a significant difference in unweighted UniFrac (ANOSIM R= 0.18, p-value = 0.006) and weighted UniFrac distances (ANOSIM R= 0.16, p-value = 0.004) between asymptomatic and symptomatic kauri soils.

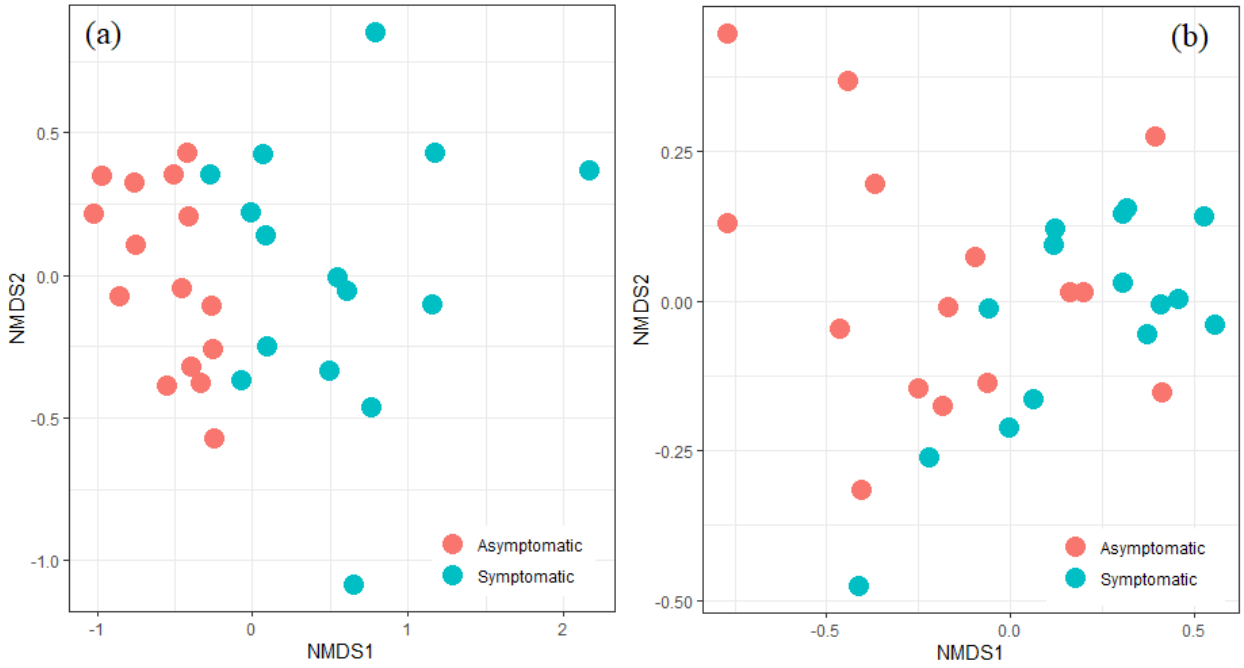

Figure 4.1. Ordination plots built using Non-metric Multidimensional Scaling (NMDS) of Bray–Curtis dissimilarity matrices that show the differences in fungal (a) and bacterial (b) community composition between asymptomatic and symptomatic kauri soils.

4.4.2 No significant differences in soil chemical properties between asymptomatic and symptomatic kauri soils

Mantel tests found no significant correlation (p-value > 0.05) between the soil chemical properties of asymptomatic and symptomatic kauri soils and their differences in fungal or bacterial community composition. Additionally, no significant differences (p-value > 0.05) were found for any of the soil chemical properties between asymptomatic and symptomatic kauri soils (Table 4.2). Although non-significant, the values for total C, organic matter, total N, bioavailable N, pH and Olsen P were found higher in symptomatic kauri soils than asymptomatic kauri soils (Table 4.2). Values for volume weight and C: N ratio were found higher in asymptomatic soils than symptomatic soils.

Table 4.2. The mean ± standard error values of the soil chemical properties measured for in symptomatic and asymptomatic soils. The results of Kruskal–Wallis chi-squared tests are displayed which were used to test for significant differences in soil chemical properties between asymptomatic and symptomatic soils.

Soil chemical property	Symptomatic	Asymptomatic	Significance
pH	5.33 ± 0.08	5.23 ± 0.09	H-value = 0.55, p-value = 0.46
Olsen P (mg/L)	6.50 ± 1.19	5.75 ± 0.48	H-value = 0.09, p-value = 0.76
Volume weight (g/mg)	0.57 ± 0.04	0.63 ± 0.05	H-value = 1.33, p-value = 0.25
Bioavailable N (ug/g)	215.75 ± 8.59	207.25 ± 16.13	H-value = 0.08, p-value = 0.77
Organic matter (%)	31.95 ± 3.18	25.70 ± 2.97	H-value = 2.11, p-value = 0.15
Total C (%)	18.53 ± 1.84	14.90 ± 1.72	H-value = 2.11, p-value = 0.15
Total N (%)	0.70 ± 0.08	0.54 ± 0.08	H-value = 1.33, p-value = 0.25
C:N ratio	27.10 ± 1.56	27.78 ± 0.59	H-value = 0.33, p-value = 0.56

4.4.3 Differences in taxonomic abundances of microbial communities between asymptomatic and symptomatic kauri soils

Fungal communities in asymptomatic and symptomatic kauri soils were dominated by the phyla Basidiomycota and Ascomycota, which contributed to over 80% relative abundance. The Tremellomycetes were a dominant class in asymptomatic kauri soils (Figure 4.2) with a relative abundance of 40% which was significantly higher than in symptomatic soil (p-value < 0.001). In symptomatic kauri soils, the Agaricomycetes were a dominant class and had a relative abundance of 41% which was significantly higher than in asymptomatic kauri soil (p-value < 0.001).

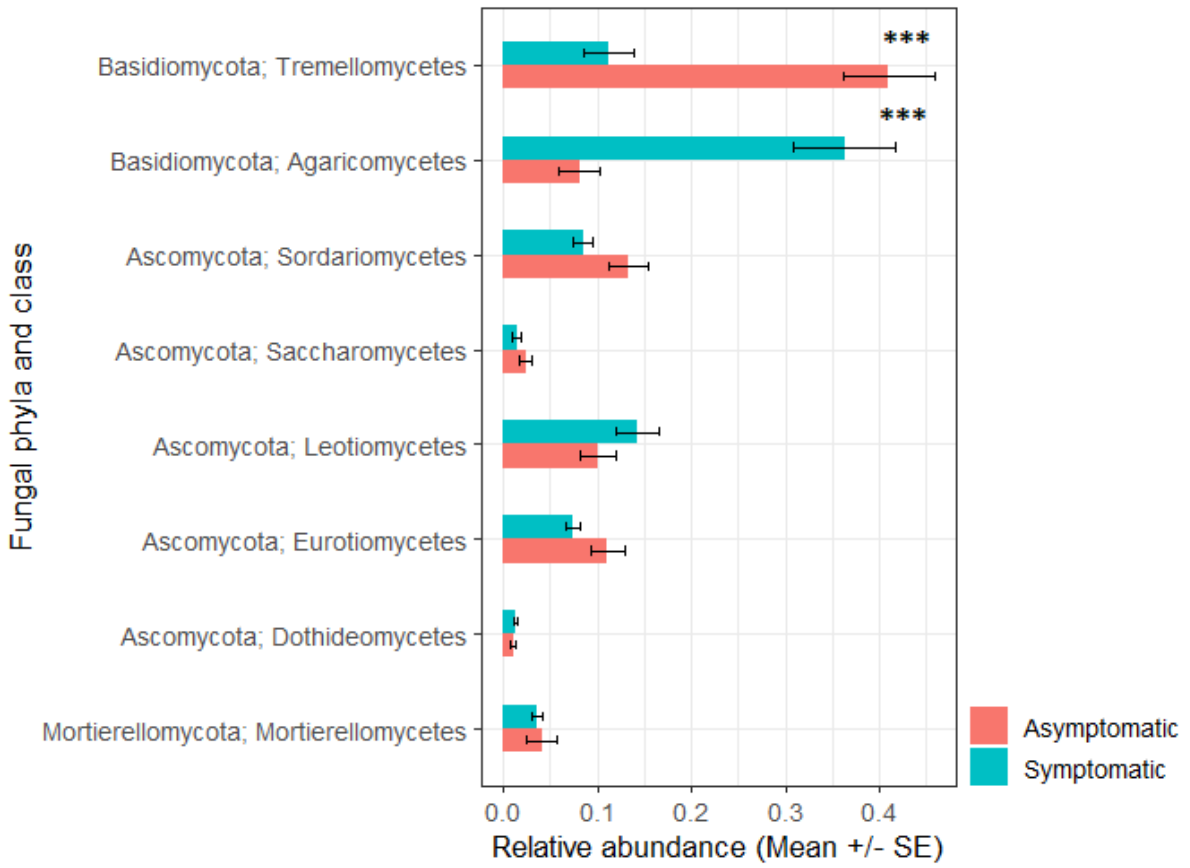

Figure 4.2. The mean ± standard error relative abundances (%) of fungal classes in asymptomatic and symptomatic kauri soils. Significant differences in the relative abundances of fungal classes between asymptomatic and symptomatic soils were determined using Kruskal-Wallis chi squared tests and are denoted by *, where p-value < 0.05 is *, p-value < 0.01 is ** and p-value < 0.001 is *.**

In asymptomatic kauri soils, 39 fungal OTUs were found in significantly higher relative abundance than in symptomatic kauri soil (p-value < 0.05). As shown in Figure 4.3, fungal OTUs with the greatest differential abundance in asymptomatic soils included the genera *Penicillium, Trichoderma, Auricularia, Backusella, Absidia, Apiotrichum* and *Barnettozyma.* In addition, several unidentified fungal genera belonging to the families Trichosporonaceae and Tricholomataceae were found in significantly higher relative abundance in asymptomatic kauri soils.

In symptomatic kauri soils, 202 fungal OTUs were found in significantly higher relative abundance compared to asymptomatic kauri soils (p-value < 0.05). As shown in Figure 4.3, fungal OTUs with the greatest differential abundance included the genera *Hygrocybe*, *Cystolepiota*, *Luellia*, *Cuphophyllus*, *Trechispora* and *Clavulinophyllus*. Many of the unidentified fungal genera found significantly higher in symptomatic soils were those belonging to the class Agaricomycetes. Only fungal OTUs identified to family and genus level are shown in Figure 4.3 and the results of the DESeq2 analysis can be seen in Table B.4, Appendix B.

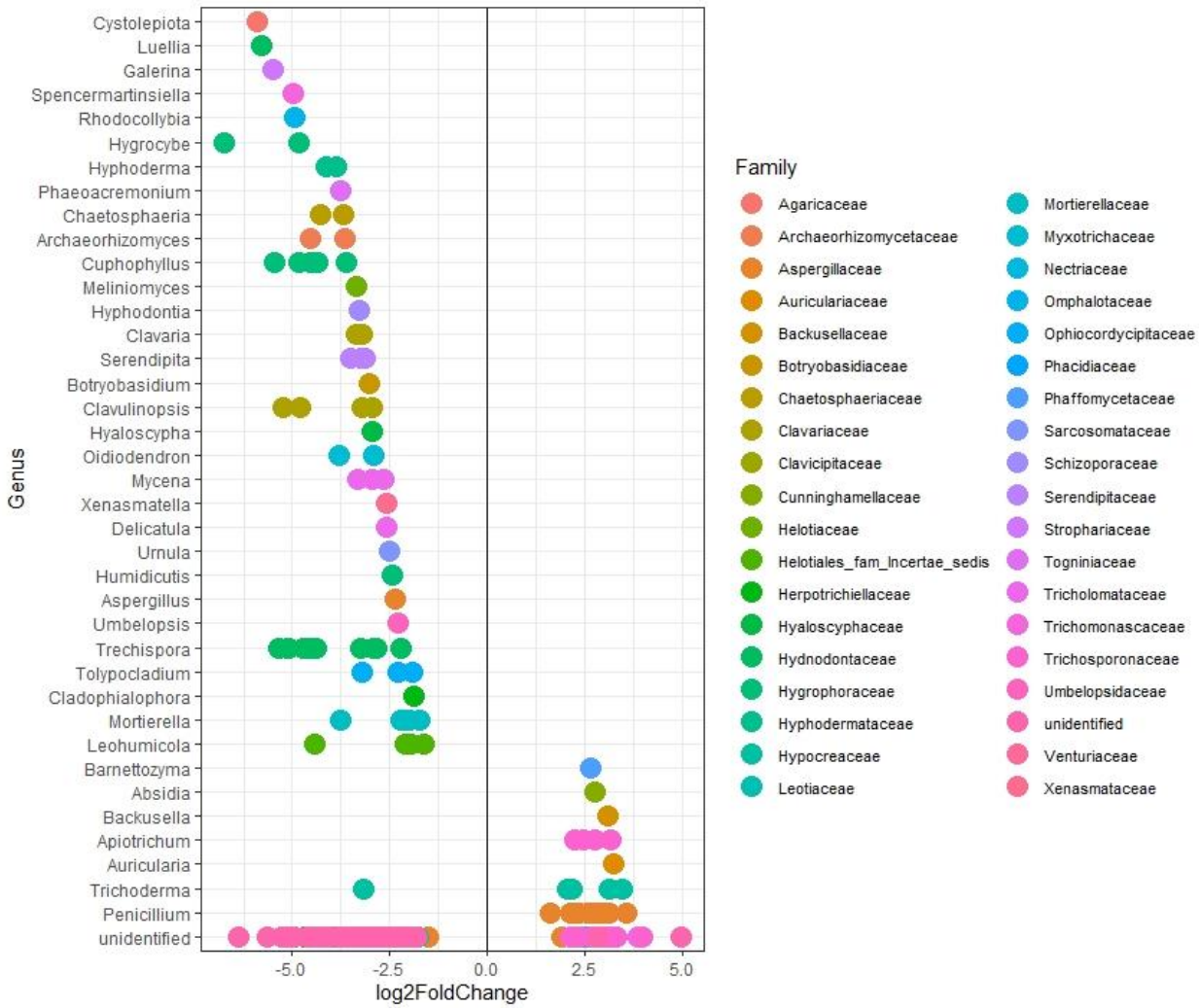

Figure 4.3. The fungal OTUs found to have significantly different relative abundances in soils from asymptomatic and symptomatic kauri. Fungal OTUs with positive 'log2FoldChange' values were found significantly higher in asymptomatic kauri soils and fungal OTUs with negative 'log2FoldChange' values were found significantly higher in soils from symptomatic kauri soils.

The phylum Proteobacteria and its class the Gammaproteobacteria were found in significantly higher relative abundance in asymptomatic kauri soils than symptomatic kauri soils (p-value < 0.05). In symptomatic kauri soils, the Acidobacteria (p-value < 0.001), Actinobacteria (p-value < 0.05), Acidimicrobiia (p-value < 0.01) and Solibacteres (p-value < 0.05) were found in significantly higher relative abundance compared to asymptomatic soils (Figure 4.4). Dominant bacteria common to both asymptomatic and symptomatic kauri soils included members of the Rhizobiales (Bradyrhizobiaceae, *Rhodoplanes*) and Rhodospirialles (Acetobacteraceae, Rhodospirillaceae).

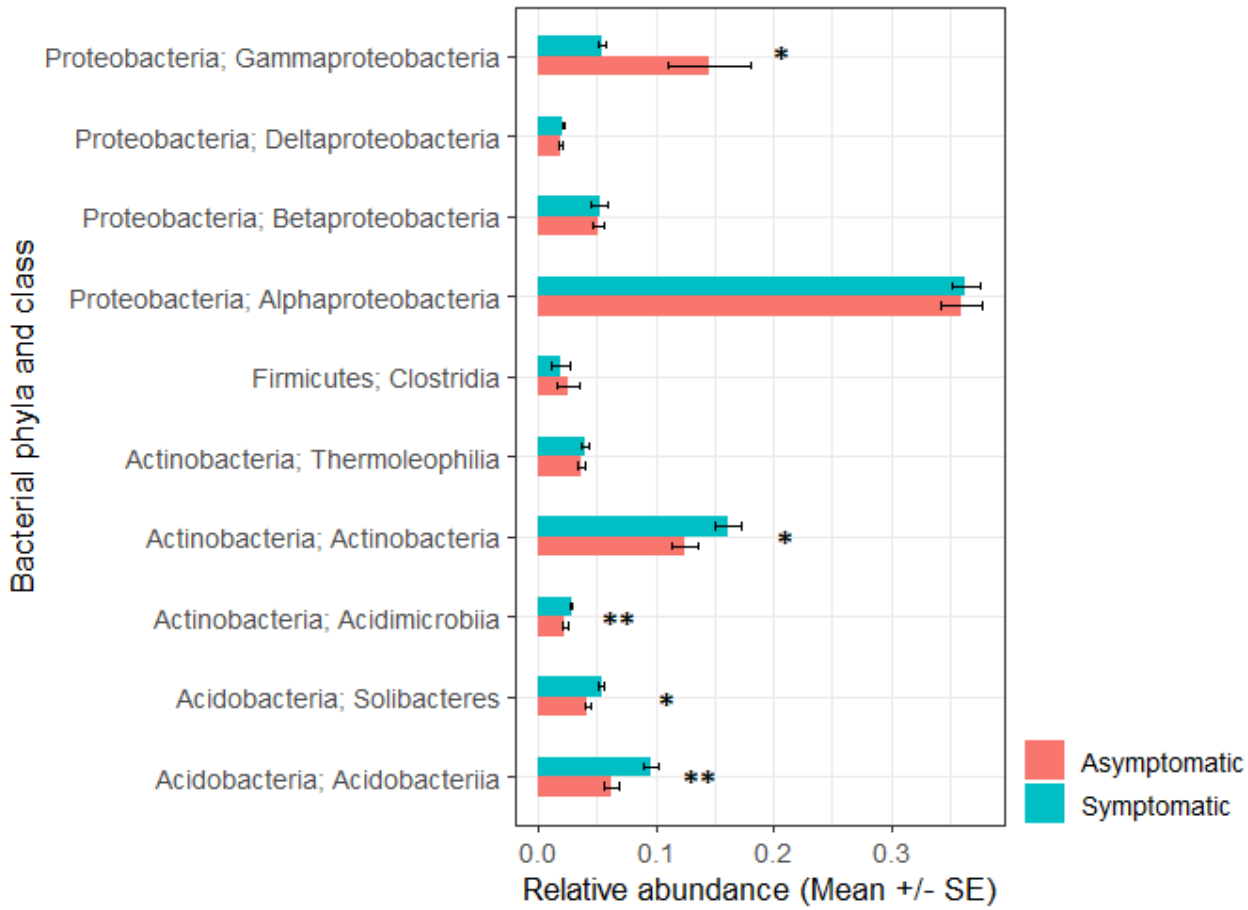

Figure 4.4. The mean ± standard error relative abundances (%) of bacterial classes in asymptomatic and symptomatic kauri soils. Significant differences in the relative abundances of bacterial classes between asymptomatic and symptomatic soils were determined using Kruskal-Wallis chi squared tests and are denoted by *, where p-value < 0.05 is *, p-value < 0.01 is ** and p-value < 0.001 is *.**

In asymptomatic kauri soils, 131 bacterial OTUs were found in significantly higher relative abundance. As shown in Figure 4.5, bacterial OTUs with the greatest differential abundance in asymptomatic soils included the genera *Acinetobacter*, *Pseudomonas*, *Flavobacterium*, *Erwinia* and *Sphingomonas.* In addition, several unidentified bacterial genera belonging to the family

Enterobacteriaceae were found in significantly higher relative abundance in asymptomatic kauri soils.

In symptomatic kauri soils, 68 bacterial OTUs were found in significantly higher relative abundance compared to asymptomatic kauri soils. As shown in Figure 4.5, bacterial OTUs with the greatest differential abundance included genera such as *Opitutus, Mycobacterium, Rhodoplanes* and *Telmatospirillum*. In addition, unidentified bacterial genera belonging to the families Koribaceraceae, Clostridiaceae and Syntrophobacteraceae were found in significantly higher relative abundance in symptomatic kauri soils. Only bacterial OTUs identified to family and genus level are shown in Figure 4.5 and the results of the DESeq2 analysis can be seen in Table B.5, Appendix B.

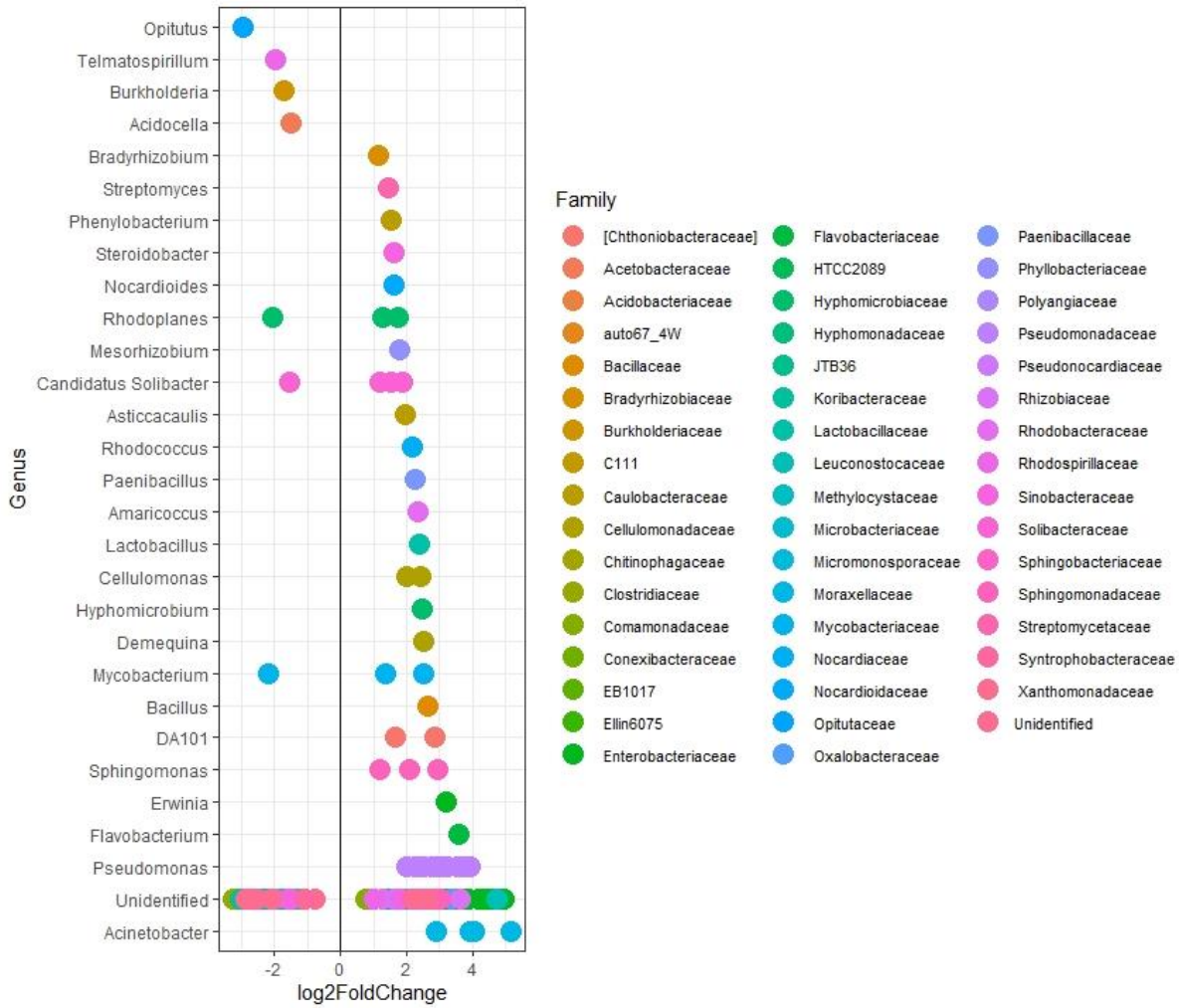

Figure 4.5. The bacterial OTUs found to have significantly different relative abundances in soils from asymptomatic and symptomatic kauri. Bacterial OTUs with positive 'log2FoldChange' values were found significantly higher in asymptomatic kauri soils and bacterial OTUs with negative 'log2FoldChange' values were found significantly higher in soils from symptomatic kauri soils

4.4.5 Changes in composition and abundance of microbial genes related to carbon and nitrogen cycling

No significant differences were found in the number of detected probes, Shannon diversity or Pielou's evenness between asymptomatic and symptomatic kauri soil (Table B.3, Appendix B). Significant differences were detected in the composition of carbon cycling (ANOSIM R= 0.304, p-value = 0.019) and nitrogen cycling genes (ANOSIM R= 0.304, p-value = 0.025) between asymptomatic and symptomatic kauri soils (Figure 4.6).

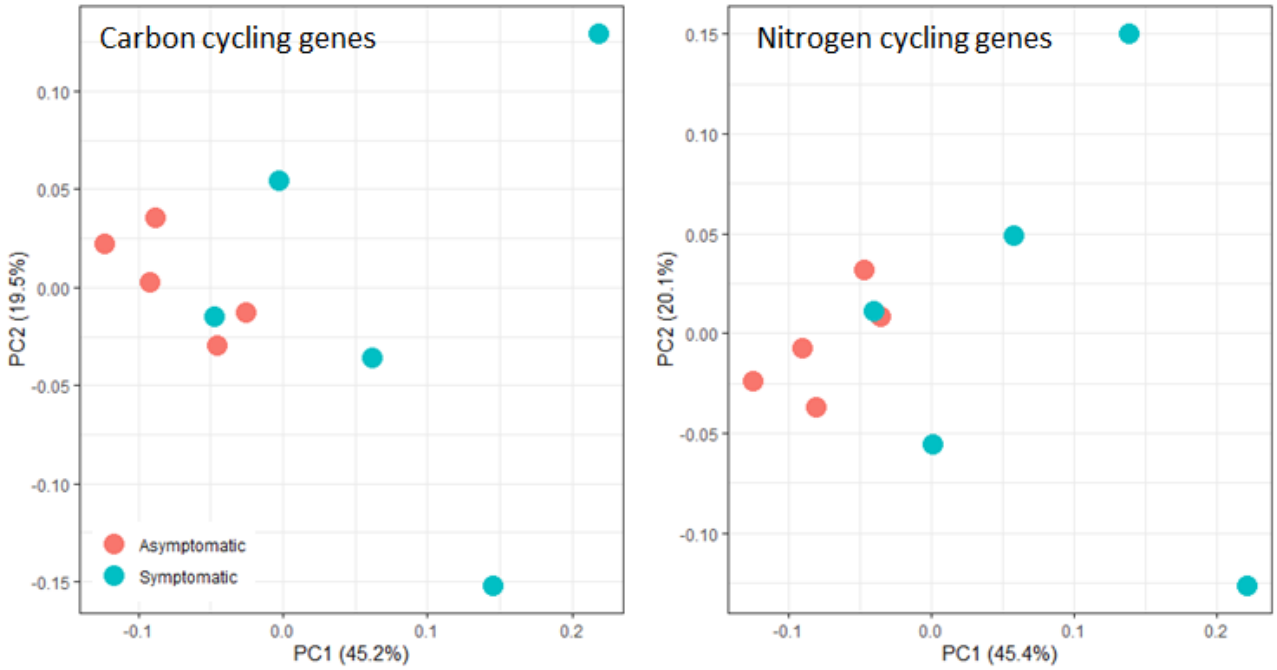

Figure 4.6. An ordination plot of the principle co-ordinates analysis (PCoA) performed using Bray–Curtis dissimilarity matrices which shows the differences in composition of carbon and nitrogen cycling genes between asymptomatic and symptomatic kauri soils.

In total, 17223 carbon cycling and 5018 nitrogen cycling gene probes were detected by the GeoChip 5S microarray. DESeq2 analysis identified that 518 of these genes had a significant differential abundance between asymptomatic and symptomatic kauri soils (p-value < 0.05). The full results of the DESeq2 analysis can be seen in Table B.6, Appendix B. A greater number of carbon degradation genes were found significantly higher in symptomatic soils (182 carbon degradation genes) than asymptomatic kauri soils (99 carbon degradation genes). Of the 120 nitrogen cycling gene probes found significantly different between asymptomatic and symptomatic soils, 47 were higher in asymptomatic soils and 73 were higher in symptomatic soils. Gene probes that had the highest differential abundance in asymptomatic kauri soil included those for pectin degradation (*RgaE*), chitin degradation (*acetylglucosaminidase*), tannin degradation (*tannase_Cdeg*), cellulose

degradation (*cellobiase*), starch (*amyA*) and hemicellulose degradation (*xyla*). Gene probes with the highest differential abundance in symptomatic soils included those for the reductive tricarboxylic acid cycle (*frdA_rTCA*), ammonification (*gdh*), cellulose degradation (*exoglucanase*), hemicellulose degradation (*xylanase*), lignin degradation (*phenol_oxidase*), agar degradation (*beta_agarase*) and dissimilatory N reduction (*nrfa*). Although many genes had significantly different abundances between asymptomatic and symptomatic soils, when genes were viewed according to their functional gene category there were no clear trends showing a split of gene categories between asymptomatic and symptomatic soils (Figure 4.7).

Figure 4.7. The carbon and nitrogen cycling genes that had a significant difference in counts between asymptomatic and symptomatic kauri soils. Genes with a positive 'log2FoldChange' value were found in significantly higher abundance in asymptomatic soil, genes with negative values were found in significantly higher abundance in symptomatic soil.

4.5 Discussion

4.5.1 Changes in microbial diversity in response to tree disease expression

This study found pronounced and significant differences in the diversity, composition and functional properties of microbial communities between asymptomatic and symptomatic kauri soils. Symptomatic kauri soils had a significantly higher fungal diversity in comparison to asymptomatic kauri soils which could be the result of a secondary colonisation processes which has been described following tree death (Jung et al., 2018). The colonisation of saprophytic fungi and secondary pathogens can occur due to the increased availability of dead plant tissues provided by the diseased host. As fungi vary in their ability to exploit resources and occupy niche space, the secondary colonisation of decayed resources can lead to changes in community composition (Boddy & Hiscox, 2017; Jung et al., 2018). Furthermore, the secondary colonisation of diseased hosts by saprophytic fungi can aid disease progression by degrading host tissue to release resting structures of *Phytophthora* that are growing within (Jung et al., 2018).

The differences in microbial community composition between asymptomatic and symptomatic kauri soils may link to differences in functional composition. Functional differentiation can explain differences in microbial diversity patterns because microbial taxa exhibit differences in their niche patterns and life history strategies (Schimel & Schaeffer, 2012). Changes to plant inputs can influence microbial community structure because microorganisms vary in their ability to decompose different types of plant material (van der Wal et al., 2013). There was a clear shift observed between the Tremellomycetes dominated asymptomatic kauri soil and the Agaricomycetes dominated symptomatic kauri soil. The Agaricomycetes are a class of mushroom forming fungi which function as saprophytic wood decay fungi in forest ecosystems and form a critical role in organic matter decomposition (Hibbett et al., 2014; Rosa & Capelari, 2009). Given their increased dominance in symptomatic kauri soil, in line with the greater number of carbon degradation genes found in significantly higher abundance in symptomatic kauri soil, they may be exhibiting a response to the increased volume of necrotic plant tissue following tree disease.

4.5.2 The microbial taxa of interest for their roles in soil disease suppression

One aim of studying the soils associated with asymptomatic kauri was to identify if they exhibited any properties of disease suppression mediated by their microbial communities. These results have identified several genera that predominated in asymptomatic kauri soils which have been studied elsewhere for their antagonistic activities against pathogens, such as *Penicillium*, *Trichoderma* and

Pseudomonas (Garbeva et al., 2004). These may represent taxa associated with good kauri health and potential disease suppressive properties.

Penicillium is a cosmopolitan genus well adapted to the soil environment, making it a highly competitive fungus capable of suppressing plant pathogens (Nicoletti & De Stefano, 2012). Numerous *Penicillium* species have reported antagonistic activities against a range of soil-borne *Phytophthora* pathogens (Fang & Tsao, 1995; Ma et al., 2008). Fang and Tsao (1995) demonstrated that *Penicillium funiculosum* reduced the symptoms of *Phytophthora* root rot and promoted growth of azalea and citrus following infection with *P. cinnamomi*, *P. parasitica* and *P. citrophthora*. Additionally, Wakelin et al. (2006) used dual culture assays to identify that *Penicillium radicum* was highly effective at inhibiting *P. cinnamomi.* Ma et al. (2008) identified *Penicillium striatisporum* to effectively inhibit the growth of *P. capsici, P. infestans, P. drechsleri, P. megasperma* and *P. nicotianae*. In the same study, *P. capsici* infested soils that were amended *P. striatisporum* had a significantly reduced incidence of root rot in chilli peppers, which as attributed to *P. striatisporum*'s production of anti-fungal metabolites.

Trichoderma, which is perhaps the most well studied and widely applied biocontrol fungus (Schuster & Schmoll, 2010) was also found in significantly higher relative abundance in asymptomatic kauri soil. Previous studies have demonstrated *Trichoderma* species to antagonise numerous different *Phytophthora* pathogens. For example, Ahmed et al. (2000) identified *T. harzianum* to reduce disease incidence in pepper plants following infection with *P. capsici* by inducing the plant defence responses. Additionally, Bae et al. (2004) found that metabolites extracted from *T. atroviride/petersenii* and *T. virens* strongly inhibited the mycelial growth of *P. melonis, P. cactorum, P. drechsleri, P. sojae, P. capsici, P. nicotianae* and *P. infestans*. Furthermore, treatment of pepper leaves with the metabolites of *T. atroviride/petersenii* inhibited the growth of *P. capsici* by inducing plant defence responses. Widmer (2014) identified two *T. asperellum* soil isolates which were able to mycoparasitise *P. ramorum* and could be used to successfully remediate *P. ramorum* infested potting mixes.

Several members of the Gammaproteobacteria were found in significantly higher relative abundance in asymptomatic kauri soil; members of this bacterial class have previously been associated with disease suppressive soils (Mendes et al., 2011). Members of the genus *Pseudomonas* have been extensively studied for their disease suppressive properties, especially against oomycete plant pathogens (Haas & Défago, 2005). Several studies have confirmed the antagonistic effects of *Pseudomonas* against *Phytophthora* pathogens, including *P. capsici* and *P. infestans* (Caulier et al., 2018; Zohara et al., 2016). Strains of *Pseudomonas fluorescens* have been shown to produce

biosurfactants that can antagonise *Phytophthora* sporangia and zoospore structures whilst promoting induced systemic resistance in plants (Tran et al., 2007). Sang and Kim (2014) identified two strains of *Pseudomonas corrugata* that were highly effective root colonisers, thus could competitively exclude *P. capsici* root colonisation and reduce plant disease severity.

Two other members of the Gammaproteobacteria, *Enterobacteriaceae* and *Acinetobacter* were also found in significantly higher abundance in asymptomatic kauri soil. In a previous study, Liu et al. (2020) isolated strains of *Enterobacter* and *Acinetobacter* which were suppressive against the pathogen *Phytophthora nicotianae*. Additionally, *Enterobacter cloaceae* has been identified to suppress disease caused by the oomycete pathogen, *Pythium ultimum*, by competing for plant derived fatty acids required by *P. ultimum* for sporangia formation (van Dijk & Nelson, 2000). In addition, *A. baumannii* has been demonstrated to inhibit *P. capsici* growth through the production of anti-fungal metabolites (Liu et al., 2007; Xue et al., 2009). These findings provide valuable guidance for the search of potential microbial antagonists against *P. agathidicida*, however more targeted studies are needed to assess the biocontrol potential of these microbial taxa against kauri dieback.

4.5.3 The functional responses of soil microbial communities

A series of secondary impacts have been observed to follow the widespread dieback of a large keystone tree species, including reduced carbon uptake, soil nutrient loss, increased plant biomass decomposition and reduced release of plant root exudates (Edburg et al., 2012). Long term these impacts can cascade to alter biogeochemical processes such as C and N cycling, plant species composition and plant productivity (Edburg et al., 2012; Schimel & Schaeffer, 2012). New Zealand's kauri forests are incredibly carbon dense, with kauri contributing to most of their forest's carbon inputs (Macinnis-Ng & Schwendenmann, 2015). Forest dieback events often result in forests transitioning from net C sinks to C sources, due to increased C losses and reduced C uptake (Avila et al., 2016). This study found significant differences in the composition of microbial genes related to carbon cycling between asymptomatic and symptomatic kauri soils, including differences in the abundances of genes related to carbon degradation. These differences may reflect a response of the soil microbial communities' changes in litter quantity and quality following tree dieback. Although non-significant, values for total C and organic matter were higher in symptomatic soils compared to asymptomatic soils, which may suggest they have increased rates of C turnover. During the degradation of organic matter, soil microorganisms release enzymes to break down complex plant carbon polymers into more simple forms for cell uptake and metabolism (Adetunji et al., 2017). Depending on the carbon substrates available and the ability of soil microorganisms to produce the appropriate enzyme, different taxonomic groups will specialise on particular substrates (i.e. sucrose,

cellulose or lignin). Additionally, the products of enzymatic breakdown can attract opportunistic microorganisms which further influence the composition and function of resident microbial communities (Schimel & Schaeffer, 2012). As discussed, a secondary colonisation process can occur following tree dieback which leads to an increased abundance of saprophytic fungi that degrade necrotic plant tissues (Jung et al., 2018). During this colonisation process, saprophytic fungi first metabolise more simple carbon forms provided by the decayed plant tissues before then utilising more recalcitrant carbon forms (Boddy & Hiscox, 2017). Symptomatic kauri soils had a greater number of carbon degradation genes found in significantly higher relative abundance than asymptomatic soils. When considered alongside the shifts in fungal community composition and the higher values of total C in symptomatic soils, these findings may suggest an increase in carbon degradation in symptomatic soils.

Kauri forest soils have large stores of immobilised nitrogen and low rates of nitrification which is attributed to their acidic pH and the slow decomposition rate and high tannin content of their leaf litter (Wyse et al., 2014). Therefore, changes to nitrogen cycling dynamics following tree dieback could potentially produce large secondary effects on the surrounding soil and plant environment. This study found significant differences in the composition of nitrogen cycling genes between asymptomatic and symptomatic kauri soils, as well as significant differences in the abundances of individual genes related to nitrogen cycling. However, in regard to N cycling there were few clear trends shown by the data to support any conclusions about what impact tree dieback may have on microbial gene function.

4.5.4 Study limitations and considerations

Previous studies have found soil N concentrations to increase following tree dieback, in response to increased inputs of N-rich litter and reduced plant N uptake (Edburg et al., 2012; Xiong et al., 2011). A kauri dieback study conducted by Schwendenmann and Michalzik (2019) in regenerative kauri forests found that increased concentrations of *P. agathidicida* DNA in kauri soils was correlated to decreased soil C and N concentrations. In this present study conducted in mature kauri forests across Waipoua Forest, symptomatic soils were found to have higher, although non-significant, C and N values compared to asymptomatic kauri soils. Differences in the findings of Schwendenmann and Michalzik (2019) and this present study could be attributed to differences in forest site, age and soil chemical testing methods. Furthermore, the non-significant differences in soil chemical properties between asymptomatic and symptomatic kauri could also be due to the time period since disease expression was observed. Typically, the impacts of root and collar rot diseases progress more gradually than other forms of tree mortality such as drought and fire, meaning their associated

short-term impacts are relatively much smaller (Anderegg et al., 2013). However over time, changes to forest species composition following selective tree death can lead to large alterations in C and N cycling dynamics across forests (Lovett et al., 2010). Indeed, findings from this study have shown significant shifts in the composition of C and N cycling genes between asymptomatic and symptomatic kauri soils. Assessing the impacts of forest disturbances on soil health and functioning using biological indicators, such as changes to microbial composition and function, can be a more sensitive method to detect short term impacts rather than measuring changes to soil physicochemical properties (Adetunji et al., 2017). Over time the changes to soil microbial diversity and function following disease outbreaks, coupled with changes to plant species composition, can cascade to impact their chemical properties such as pH, total C, total N and organic matter content (Edburg et al., 2012).

4.6 Conclusion

This study has found significant differences in the composition of fungal and bacterial communities between asymptomatic and symptomatic soils, and fungal diversity was identified to be significantly higher in symptomatic soils. The results of taxonomic analyses identified several microbial taxa significantly higher in asymptomatic kauri soil such as *Penicillium, Trichoderma, Enterobacteriaceae, Acinetobacter* and *Pseudomonas*. Several of these taxa are well studied in the literature for their roles in plant health and disease suppression. Such findings warrant further research that should aim to isolate and screen these microbial taxa against *P. agathidicida* to assess their capabilities as potential biocontrol agents. In addition, the plant beneficial roles that these microbial taxa may possess should be explored, so that microbial taxa which can support kauri health in the face of pathogen attack can begin to be identified.

4.7 Acknowledgements

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Chapter 5

The response of the soil microbial communities to the infection of kauri (*Agathis australis*) seedlings with *Phytophthora agathidicida*.

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Supplementary material for this chapter is provided in Appendix C.

5.1 Abstract

New Zealand's ancient kauri (*Agathis australis*) forests are under threat from the spread of dieback disease, caused by the soil-borne pathogen *Phytophthora agathidicida*. Characterising the response of the soil microbiota to the infection of kauri with *P. agathidicida* is essential to identify how they may form a protective response to pathogen invasion and disease expression. This study infected 18-month-old kauri seedlings with a standardised inoculum load of *P. agathidicida* for six weeks under controlled environmental conditions. Following this, changes in the diversity, composition and biomass of soil microbial communities associated with kauri seedlings were characterised using high throughput 16S rRNA and ITS gene region sequencing and phospholipid fatty acid analysis. Significant differences were found in the composition of soil bacterial communities between inoculated and non-inoculated kauri seedlings. Furthermore, soils of inoculated seedlings had a significantly higher relative abundance of bacteria known to be associated with plant disease suppression, which included several members of the Firmicutes. Significant reductions were found in the fungal: bacterial biomass of soils from inoculated seedlings, which contrasts to previous field-based research that observed an increased diversity of soil fungal communities associated with symptomatic kauri in old growth kauri forests. Further research that aims to isolate members of kauri's soil microbiota and study their interactions with *P. agathidicida* is required for us to begin selecting potential biocontrol agents against kauri dieback.

5.2 Introduction

The spread of kauri dieback, caused by the soil-borne pathogen *Phytophthora agathidicida*, threatens the survival of New Zealand's remnant kauri (*Agathis australis*) forests (Beever et al., 2009; Black et al., 2018). The soil microbiota have numerous functional roles in supporting plant health and providing defence against soil-borne pathogens (Berendsen et al., 2012). Several properties of soil microbiota, such as their biological and functional diversity, taxonomic composition, biomass and activity can influence their ability to suppress pathogens (Bonanomi et al., 2014). The interactions between invading pathogens and the resident microbiota can determine the outcome of disease progression, with resident microorganisms that establish antagonistic interactions being those which can successfully suppress pathogen development (Raaijmakers et al., 2009). Studying the microbial communities associated with plant disease outbreaks can identify soil microorganisms that are responding to plant attack following pathogen invasion (Galiana et al., 2011). This study aimed to characterise the changes in diversity, composition and biomass of soil microbial communities associated with kauri seedlings six weeks following their inoculation with *P. agathidicida*. By doing so, this research aimed to better understand how the kauri soil microbiota responds to the initial stages of pathogen invasion and seedling disease expression.

Previous research presented in **Chapter 4** identified significant differences in the diversity and composition of soil microbial communities associated with asymptomatic and symptomatic mature kauri across Waipoua Forest (Northland Region, New Zealand). Furthermore, the expression of kauri dieback was found to significantly impact the composition of microbial genes related to carbon (C) and nitrogen (N) cycling, with these impacts having the potential to significantly alter long term soil nutrient cycling in kauri forests. In addition, microbial taxa with previously reported roles in supporting soil disease suppression such as *Penicillium*, *Trichoderma,* and *Pseudomonas* (Garbeva et al., 2004) had a significantly higher relative abundance in asymptomatic soils compared to symptomatic soils. However, there were several methodological limitations to **Chapter 4** that require consideration. Firstly, it was not possible to sample soils from symptomatic kauri which were infected with a consistent inoculum load of *P. agathidicida*. Although the symptomatic kauri selected for sampling expressed a similar degree and range of dieback symptoms (Beever et al., 2010; Waipara et al., 2013), dieback disease expression has a variable latency period (Bradshaw et al., 2020). As a result, the time since initial tree infection could not be determined based solely off disease symptoms. In addition, it was not possible to determine that the differences in soil microbial communities were a response to pathogen invasion, or rather a response to the local environmental changes which occurred following tree dieback.

Despite all sizes and age classes of kauri being susceptible to dieback, there is a limited understanding of how the degree of dieback varies across different tree sizes and age classes (Black & Dickie, 2016; Scott & Williams, 2014). For this study, observing the response of the soil microbiota to the infection of mature kauri trees with *P. agathidicida* using controlled field experiments would have provided a more congruent follow up to **Chapter 4**. However, due to the threatened conservation status and high cultural value of kauri trees (De Lange et al., 2013; Lambert et al., 2018), infecting mature kauri using controlled field experiments was not possible. Therefore, this study infected young kauri seedlings with a standardised load of *P. agathidicida* to examine the response of the soil microbiota to infection under controlled experimental conditions. The soils of kauri seedlings were sampled six weeks following inoculation, and this short-term six-week inoculation period was selected to identify microbial taxa which were primary respondents to seedling infection. The diversity and composition of soil microbial communities associated with inoculated and non-inoculated kauri seedlings were characterised using high throughput 16S rRNA and ITS gene region sequencing. In addition, phospholipid fatty acid analysis (PLFA) was used to assess the differences in viable microbial biomass between soils from inoculated and non-inoculated seedlings (Nkongolo & Narendrula-Kotha, 2020).

5.3 Methods

5.3.1 Soil sampling

Forest soils collected from the stands of uninfected kauri trees which tested negative for the presence of *P. agathidicida* were used as potting mix for the seedling bioassay. To obtain these soils, organic layer soil samples were collected from 18 asymptomatic kauri trees across Waipoua Forest (Northland Region, New Zealand) according to the sampling methods as outlined in **Chapter 4** (**Section 4.3.1**). The GPS coordinates of kauri trees sampled across Waipoua Forest are shown in Table C.1, Appendix C. Following sampling, the presence or absence of *P. agathidicida* in kauri soils was determined using a soil baiting bioassay (Bellgard et al., 2013) and a real-time PCR assay (McDougal et al., 2014; Than et al., 2013). A full description of these methods is provided in Supplementary Methods B.1, Appendix B. Only soil samples which tested negative for *P. agathidicida* across both pathogen detection methods were used as potting mix. The results of soil pathogen bioassays can be found in Table C.2, Appendix C. The sampling locations of the soil samples which qualified for use as seedling potting mix are displayed in Figure C.1, Appendix C.

5.3.2 Seedling bioassays

For the seedling bioassays, 60 18-month old kauri seedlings approximately 20 cm in height were sourced from the Auckland Botanical Gardens (Auckland, New Zealand). Kauri seedlings were transplanted into 1 litre plant pots that were filled with 500 g of uninfected kauri soil. Following transplant, seedlings were left to equilibrate for 2 weeks and monitored for symptoms of ill health or transplant damage. Seven seedlings showed signs of transplant damage and were discarded, which left 53 seedlings for the experiment.

The *P. agathidicida* strain NZFS3770 was used as a pathogen inoculum source for the seedling bioassay. This strain was recieved from the National Forest Culture Collection (Scion, Rotorua, New Zealand) and was originally isolated in 2006 from the Coromandel, New Zealand. The full genome of *P. agathidicida* NZFS3770 has been sequenced by Studholme et al. (2016) and is available under the GenBank accession number LGTS00000000.

Cultures were maintained at 21.5^{o}C in darkness on 20% clarified V8 agar (Lawrence et al., 2017). Prior to setting up the seedling bioassay, lupin (*Lupinus angustifolius*) seedlings were infected to confirm pathogenicity of *P. agathidicida* NZFS3770 (Lewis, 2018). To prepare the inoculum source, 5 mm^2 agar plugs of *P. agathidicida* were incubated in 10 ml of 2% concentrate V8 broth in darkness at 21.5^{o}C for 24-36 hours, or until sufficient mycelial growth of 3 cm was observed (Horner & Hough, 2014; Lawrence et al., 2017). An image of a mycelial mat of *P. agathidicida* is shown in Figure C.2, Appendix C. These mats of *P. agathidicida* mycelium were used to infect 27 kauri seedlings. As a control, 26 kauri seedlings were inoculated with blank V8 agar plugs which had been incubated in V8 broth in darkness at 22^{o}C for 24-36 hours. This control was selected to ensure inoculated and non-inoculated seedlings had similar loads of nutrient rich V8 agar and broth introduced into their soils. Each seedling was inoculated with five mycelial mats, or blank V8 agar plugs, which were spaced evenly around the seedling. Mycelial mats were inserted 10 cm deep into the soil profile to be within the seedling root zone (Davison & Tay, 1987). During preliminary experiments, disease symptoms would initially appear following infection but over time these symptoms did not progress in a manner that resulted in an observable and stable degree of disease expression. To ensure sustained infection during the experiment, seedlings were re-inoculated on week two and week three with three *P. agathidicida* mycelial mats, whilst control (non-inoculated) seedlings were re-inoculated with three blank V8 agar plugs. Following inoculation, inoculated and non-inoculated seedlings were held at 100% water holding capacity for one week to promote zoospore motility of *P. agathidicida* and stimulate seedling infection (Davison & Tay, 1987). Saucers containing the seedling pots were monitored and, if necessary, drained to prevent a build-up of water. Following this, seedlings were

maintained at 50% of soil water holding capacity for the remainder of the experiment. For the duration of the experiment, seedling bioassays were incubated in growth chambers (Sanyo Versatile Environmental Test Chamber MLR-351) which were maintained at a constant temperature of 18°C (Horner & Hough, 2014) with cycling photoperiods of 12 hours light and 12 hours darkness.

Seedlings were inoculated with *P. agathidicida* for six weeks before they were destructively sampled and their soils were collected for analysis. Harvested seedlings were measured for seedling length (mm), weight (g), shoot weight (g), root weight (g) and root: shoot ratio. For each seedling, the soil within a 2 cm^2 area surrounding their root system, (~10 cm depth of the soil profile) was collected and sieved < 2 mm^2 to remove plant material and homogenise sample. For soil RNA extractions, 10 g subsamples were preserved in LifeGuard Soil Preservation Solution (Qiagen, Germany) and stored at -80°C. For soil DNA extractions, 10 g subsamples were stored at -20°C. For PLFA analysis, 10 g to 20 g subsamples were stored at 4°C.

5.3.3 Soil DNA extractions and amplicon sequencing

Soil DNA extractions and amplicon sequencing followed methods outlined in **Chapter 3** (**Section 3.3.2**). Bioinformatics analysis followed methods outlined in **Chapter 4** (**Section 4.3.4**). Briefly, DNA extractions were performed on three 0.25 g soil samples collected from each seedling. Following quality control checks, DNA extracts were sent to Novogene Co., Ltd (Hong Kong) for library preparation and sequencing of the bacterial 16S rRNA (V3-V4) and the fungal ITS (ITS2) gene region on an Illumina HiSeq platform. Initial processing and quality filtering of sequencing reads were performed using QIIME V 1.7.0 (Caporaso et al., 2010). Sequencing reads were clustered into OTUs at 99% sequence similarity using QIIME 2 2019.4 (Bolyen et al., 2019). OTU clusters were assigned taxonomies using the reference databases 'Green Genes 13.8' for 16S rRNA classification and 'UNITE 18.11.2018' for ITS classification. The Qiime 2 'diversity' plugin was used to conduct alpha and beta diversity analyses. Taxonomic differential abundance testing and visualisation was performed using the R packages Phyloseq (McMurdie & Holmes, 2013), DESeq2 (Love et al., 2014) and Vegan (Oksanen et al., 2019).

5.3.4 Phospholipid fatty acid analysis

To prepare soils for PLFA analysis, 5 to 10 g soil samples from 15 randomly selected inoculated seedlings and 15 randomly selected non-inoculated seedlings were freeze dried for 4 days. Freeze dried soil samples were sent to Microbial ID, Inc. (Newark, USA) for high throughput PLFA analysis using the Sherlock PLFA analysis system (Buyer & Sasser, 2012). Individual phospholipid fatty acids were grouped into different microbial classes based on assignments previously defined by Willers et

al. (2015). Fatty acid chains 17:1 ω7c 10-methyl, 17:0 10-methyl, 18:1 ω7c 10-methyl and 18:0 10-methyl were assigned as Actinomycetes; 16:1 ω9c, 16:1 ω7c, 17:1 ω8c, 17:0 cyclo ω7c, 18:1 ω7c, 18:1 ω5c, 19:0 cyclo ω7c and 20:1 ω9c were assigned as Gram-negative bacteria; 14:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:1 iso ω9c, 17:0 iso and 17:0 anteiso were assigned as Gram-positive bacteria; 14:0, 15:0, 16:0 and 17:0 were assigned as Other Eubacteria; 16:1 ω5c was assigned as arbuscular mycorrhizal fungi (AM Fungi); 18:1 ω9c was assigned as saprophytic fungi and 18:2 ω6c was assigned as ectomycorrhizal fungi (EM Fungi).

5.3.5 Quantification of *P. agathidicida* abundance

To quantify the abundance of *P. agathidicida* in each soil sample, real-time PCR assays were performed on soil DNA extracts performed in **Section 5.3.2**. These were performed to confirm that inoculated seedlings tested positive for *P. agathidicida* and that non-inoculated seedlings tested negative for *P. agathidicida*. The resulting cDNA samples were quantified for their abundance of *P. agathidicida* using a real-time PCR assay (McDougal et al., 2014; Than et al., 2013), the methods of which are outlined in Supplementary Materials B.1, Appendix B.

5.4 Results

5.4.1 Seedling disease expression

The results of the real-time PCR assays found that all 26 inoculated seedlings tested positive for *P. agathidicida*. At the end of the experiment, the average abundance of *P. agathidicida* cDNA detected in the soils of inoculated seedlings was 34.60 ± 4.93 femtograms. The soils of all non-inoculated seedlings tested negative for *P. agathidicida*, having undetermined Ct values and a reported *P. agathidicida* cDNA abundance of 0.00fg. The results of these real-time PCR assays are shown in Table C.3, Appendix C. Seedling disease assessments taken at the end of the experiment found that the dry root weights, shoot weights, root: shoot ratio and seedling length were significantly lower in inoculated seedlings than non-inoculated seedlings (Table 5.1). All inoculated seedlings expressed symptoms of dieback including chlorosis, wilting, leaf litter loss, necrosis and mortality (Figure C.3, Appendix C).

Table 5.1. The mean ± standard error values for each measurement used to quantify differences in seedling biomass between non-inoculated and inoculated kauri seedlings. The results of student's T-tests are displayed which were used to test for significant differences in the biomass measurements of inoculated and non-inoculated seedlings.

Seedling measurement	Inoculated	Non-inoculated	Significance
Root: Shoot ratio	0.11 ± 0.02	0.30 ± 0.02	t = 7.63, p-value < 0.001
Root weight (g)	0.17 ± 0.02	0.58 ± 0.05	t = 7.22, p-value < 0.001
Shoot weight (g)	2.05 ± 0.18	1.58 ± 0.13	t = 2.09, p-value = 0.04
Length (mm)	183.96 ± 4.99	201.46 ± 6.00	t = 2.24, p-value = 0.03

5.4.2 Differences in microbial diversity and composition

There were no significant differences in the Shannon diversity or Pielou's evenness of soil fungal communities between inoculated and non-inoculated kauri seedlings (Table 5.2). The number of observed fungal OTUs was significantly lower in inoculated seedlings compared to non-inoculated seedlings. For soil bacterial communities, the Shannon diversity, Pielou's evenness and number of observed OTUs was significantly higher in inoculated seedlings compared to non-inoculated seedlings.

Table 5.2. The mean ± standard error alpha diversity values calculated for fungal and bacterial communities in inoculated and non-inoculated seedlings. The results of Kruskal-Wallis chi squared tests are displayed which were used to test for significant differences in the alpha diversity of inoculated and non-inoculated seedlings.

	Alpha diversity	Inoculated	Non-inoculated	Significance
Fungi	**Shannon diversity**	5.39 ± 0.12	5.59 ± 0.14	H = 0.85, p-value = 0.36
	Pielou's evenness	0.44 ± 0.01	0.45 ± 0.01	H = 0.41, p-value = 0.52
	Observed OTUs	4974.40 ± 108.60	5389.81 ± 133.63	H = 4.94, p-value = 0.03
Bacteria	**Shannon diversity**	11.93 ± 0.03	11.76 ± 0.03	H = 12.41, p-value < 0.001
	Pielou's evenness	0.85 ± 0.00	0.84 ± 0.00	H = 11.91, p-value < 0.001
	Observed OTUs	17009.19 ± 211.31	16178.69 ± 171.99	H = 9.69, p-value = 0.002

The differences in fungal and bacterial community composition are displayed in Figure 5.1. There was no significant difference in the fungal community composition of soils associated with inoculated and non-inoculated seedlings (ANOSIM R = 0.050, p-value = 0.05). In contrast, there was a significant difference in the bacterial community composition of soils associated with inoculated and non-inoculated seedlings (ANOSIM R = 0.391, p-value < 0.001).

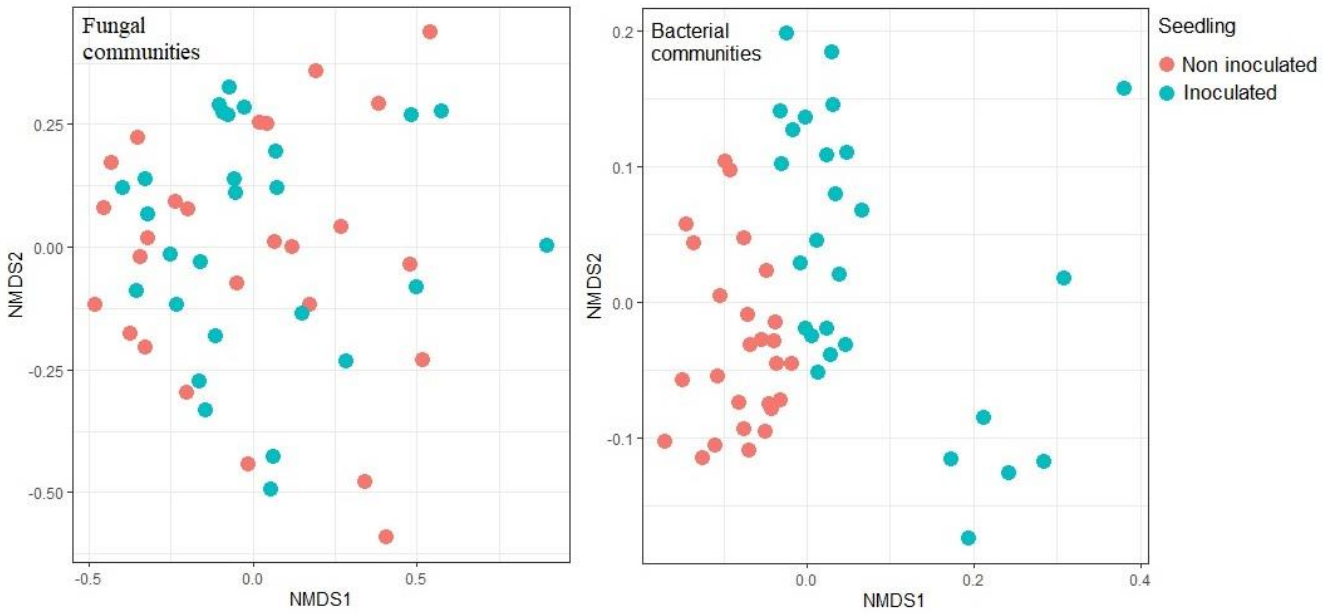

Figure 5.1. Ordination plots built using Non-metric Multidimensional Scaling (NMDS) of Bray–Curtis dissimilarity matrices which show the differences in fungal and bacterial community composition in soils associated with inoculated and non-inoculated kauri seedlings.

5.4.3 Changes in taxonomic composition of fungal communities

The phyla Mortierellomycota (H- value = 14.23, p-value < 0.001) and Rozellomycota (H-value = 7.22, p-value = 0.007) had a significantly higher relative abundance in soils from non-inoculated seedlings compared to inoculated seedlings. The Botryosphaeriales (H-value = 22.23, p-value < 0.001) had a significantly higher relative abundance in soils from inoculated seedlings, whereas the Mortierellales (H-value = 14.23, p-value < 0.001) had a higher relative abundance in soils from non-inoculated seedlings (Figure 5.2).

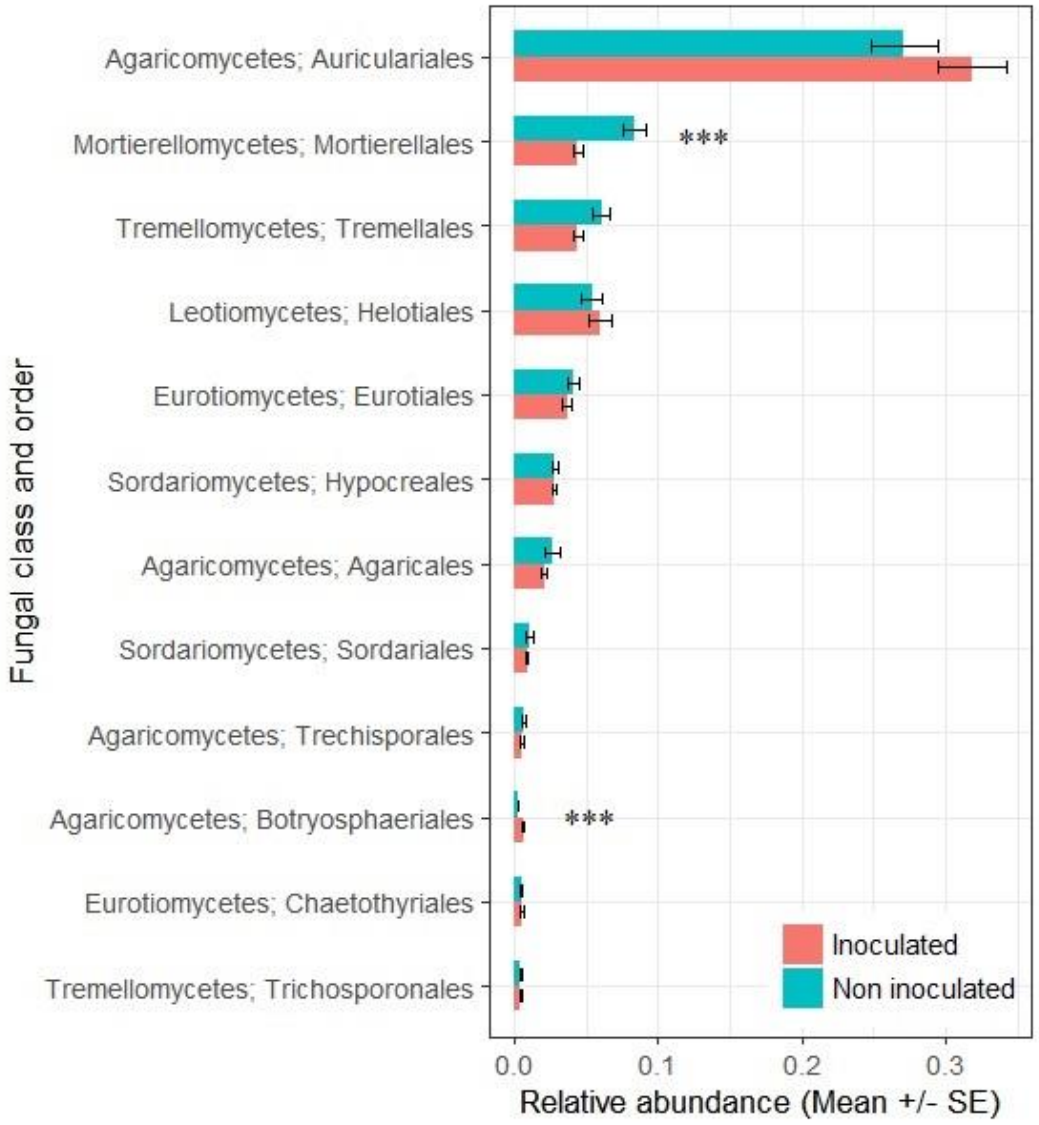

Figure 5.2. The mean ± standard error relative abundances (%) of fungal orders in soils from inoculated and non-inoculated seedlings. Significant differences in relative abundances of fungal orders were determined using Kruskal-Wallis chi squared tests and are denoted by *, where p-value < 0.05 is *, p-value < 0.01 is ** and p-value < 0.001 is *.**

As shown in Figure 5.3, 43 fungal OTUs were found in significantly higher relative abundance in soils from inoculated seedlings. These taxa included Hyaloscyphaceae, *Meliniomyces, Pezicula*, Helotiales, Auriculariales, *Leucoagaricus, Byssochlamys, Candida,* Herpotrichiellaceae, Strophariaceae, *Tubulicium, Tolypocladium,* Piskurozymaceae, *Aspergillus* and Botryosphaeriales. 20 fungal OTUs were found to have a significantly lower relative abundance in soils from inoculated seedlings which included the taxa *Mortierella,* Pyronemataceae, *Galerina, Absidia, Mucor, Neobulgaria,* Hydnodontaceae, Pseudeurotiaceae and Clavariaceae. The full results of this DESeq analysis performed on fungal OTUs is shown in Table C.4, Appendix C.

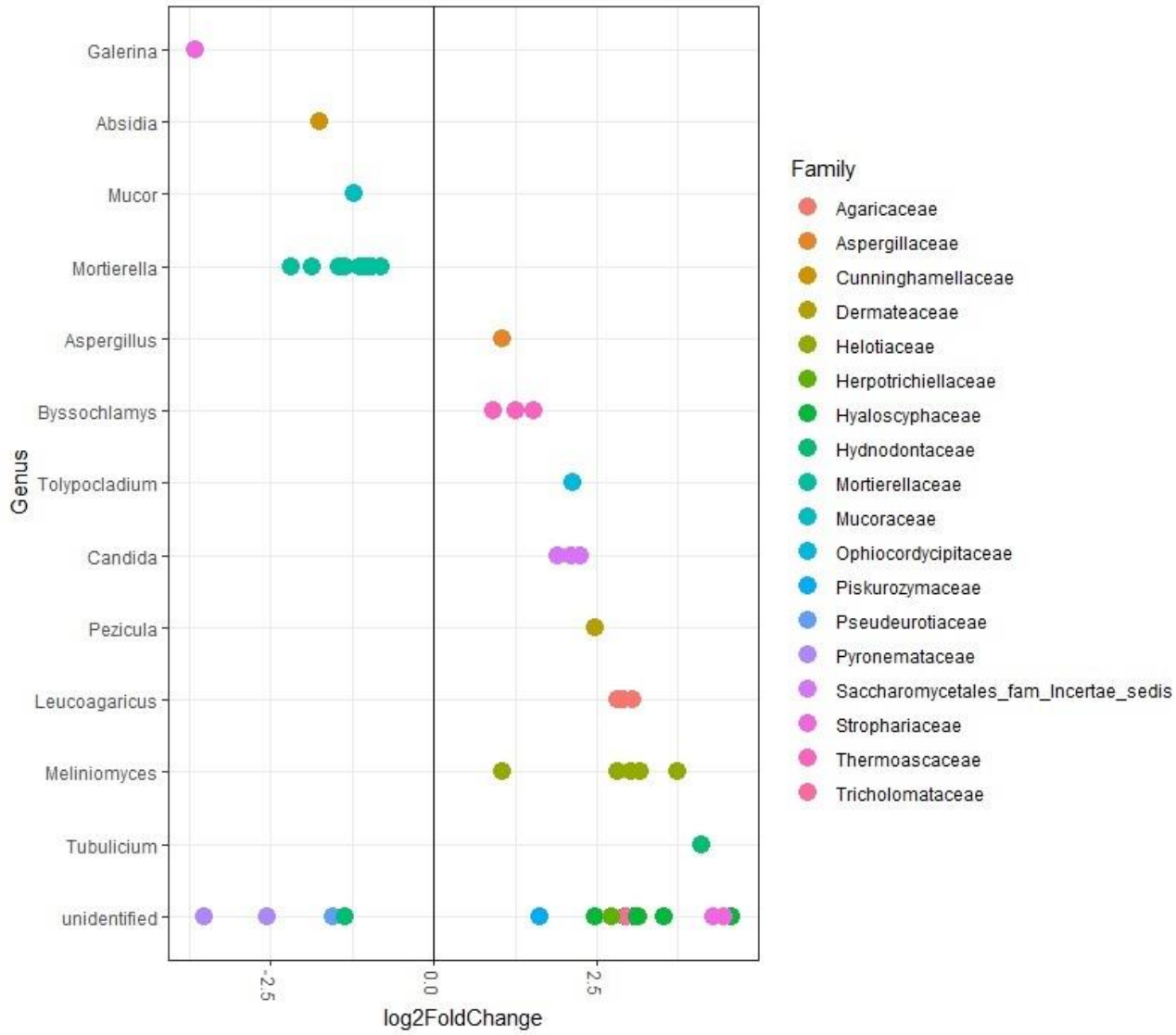

Figure 5.3. The fungal OTUs found to have significantly different relative abundances in soils from inoculated and non-inoculated kauri seedlings. Fungal OTUs with positive 'log2FoldChange' values were found significantly higher in soils from inoculated seedlings and fungal OTUs with negative 'log2FoldChange' values were found significantly higher in soils from non-inoculated seedlings.

5.4.4 Changes in taxonomic composition of bacterial communities

The phylum Firmicutes was found in significantly higher relative abundance in soils from inoculated seedlings (H- value = 39, p-value < 0.001). Actinobacteria (H- value = 25.35, p-value < 0.001), Verrucomicrobia (H- value = 6.66, p-value = 0.01) and Nitrospirae (H- value = 7.70, p-value = 0.01) were found in significantly higher relative abundance in soils from non-inoculated seedlings. As shown in Figure 5.4, bacterial orders found in significantly higher relative abundance in soils from inoculated kauri seedlings were Clostridales (H- value = 39, p-value < 0.001), Burkholderiales (H- value = 33.23, p-value < 0.001) and Bacillales (H- value = 36.40, p-value < 0.001). Bacterial orders

with a significantly higher relative abundance in soils from non-inoculated seedlings were Rhizobiales (H- value = 28.30, p-value < 0.001), Rhodospirillales (H- value = 18.08, p-value < 0.001), Solibacterales (H- value = 14.09, p-value < 0.001), Solirubrobacterales (H- value = 25.53, p-value < 0.001), Myxococcales (H- value = 7.70, p-value = 0.006), Actinomycetales (H- value = 17.33, p-value < 0.001), Acidimicrobiales (H- value = 27.92, p-value < 0.001), Gaiellales (H- value = 14.50, p-value < 0.001) and Chthoniobacterales (H- value = 8.83, p-value = 0.002).

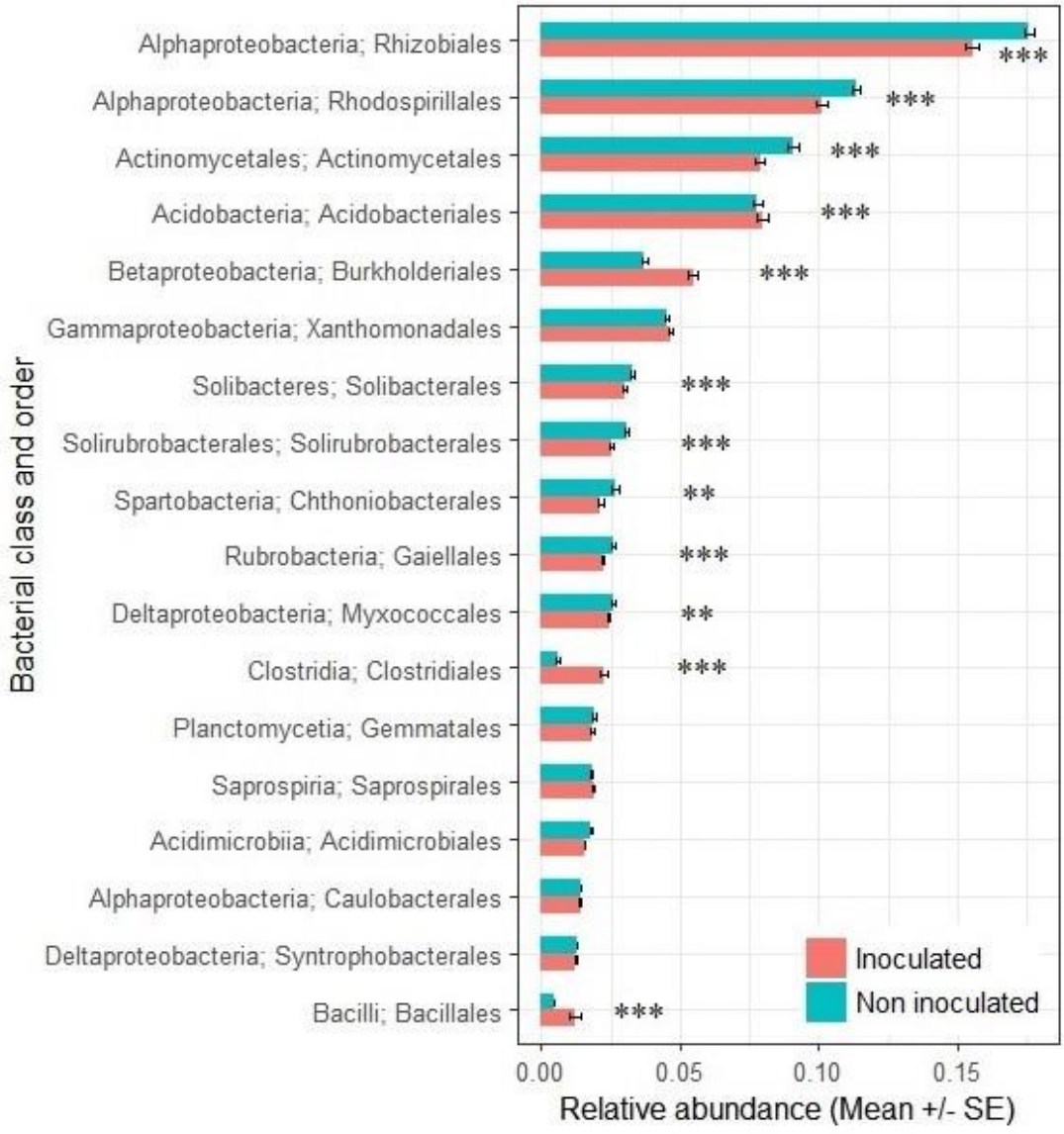

Figure 5.4. The mean ± standard error relative abundances (%) of bacterial orders in soils from inoculated and non-inoculated seedlings. Significant differences in relative abundances of bacterial orders were determined using Kruskal-Wallis chi squared tests and are denoted by *, where p-value < 0.05 is *, p-value < 0.01 is ** and p-value < 0.001 is *.**

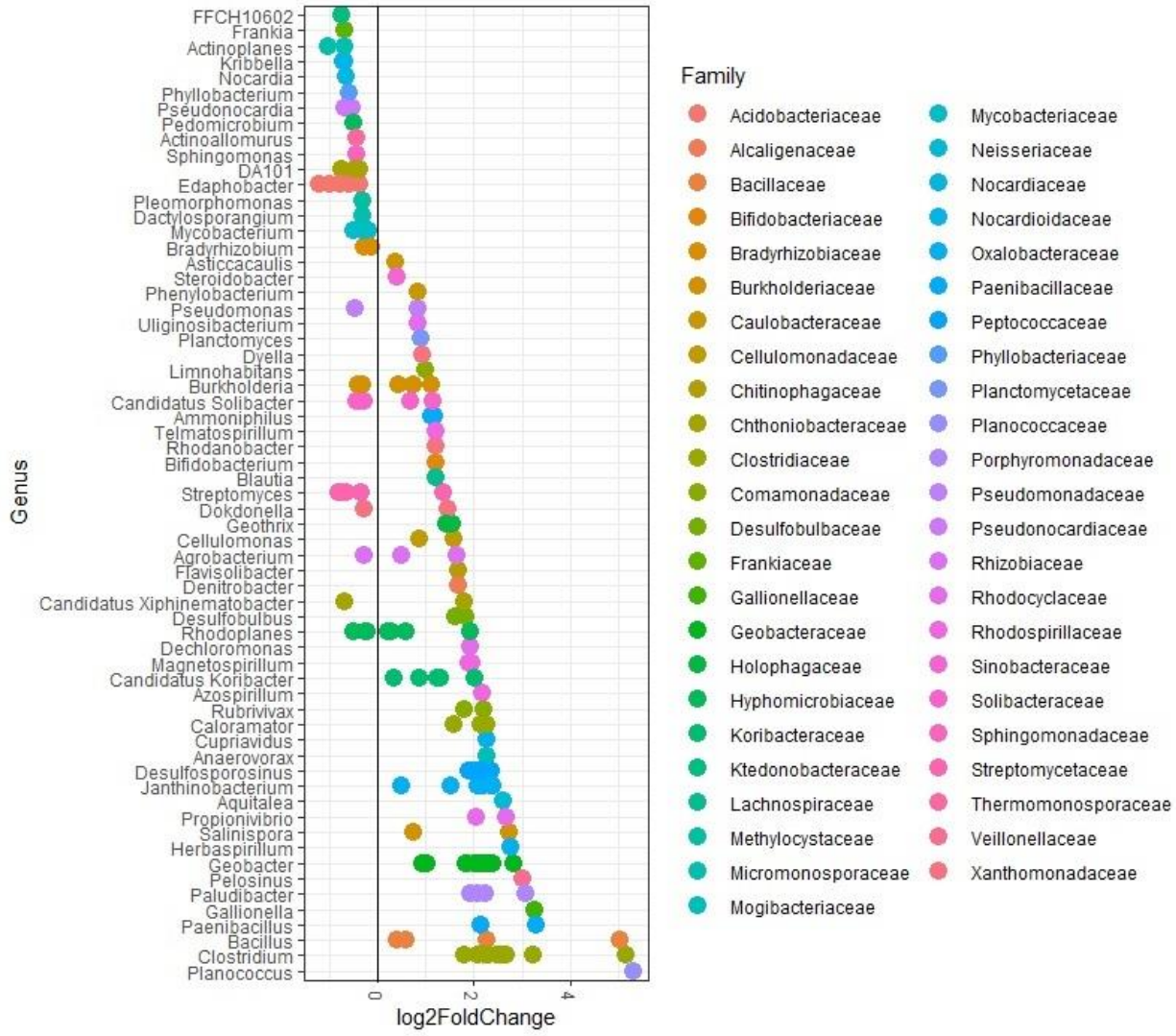

Figure 5.5. The bacterial OTUs found to have significantly different relative abundances in soils from inoculated and non-inoculated kauri seedlings. Bacterial OTUs with positive 'log2FoldChange' values were found significantly higher in soils from inoculated seedlings and bacterial OTUs with negative 'log2FoldChange' values were found significantly higher in soils from non-inoculated seedlings.

As shown in Figure 5.5, 114 bacterial OTUs were found in significantly higher relative abundance in soils from inoculated seedlings. These taxa included *Bacillus, Clostridium, Planococcus, Paenibacillus, Gallionella, Paludibacter, Geobacter, Herbaspirillum* and *Salinspora*. In total, 48 bacterial OTUs were found in significantly lower relative abundance in soils from inoculated seedlings. These taxa included *Frankia, Actinoplanes, Kribbella, Nocardia, Phyllobacterium, Pseudonocardia, Pedomicrobium, Edaphobacter* and *Sphingomonas*. The full results of the DESeq2 analysis performed on bacterial OTUs are shown in Table C.5, Appendix C.

5.4.5 Phospholipid fatty acid analysis

Soils from inoculated seedlings had a significantly higher relative abundance of total bacteria and other Eubacteria compared to soils from non-inoculated seedlings (Table 5.3). The relative abundances of total fungi, EM fungi, Actinomycetes and the fungal: bacterial ratio was significantly lower in soils from inoculated seedlings compared to non-inoculated seedlings. There were no significant differences in the total PLFA abundance (nmol/g), nor the relative abundances of Gram-positive bacteria, Gram-negative bacteria, AM fungi or saprophytic fungi, between soils from inoculated and non-inoculated seedlings.

Table 5.3. The mean ± standard error relative abundance (%) of microbial groups in soils from inoculated and non-inoculated seedlings. Values for each microbial group were calculated as the sum of each group's abundance (ng/mol) relative to the total PLFA (ng/mol). The results of Student's T tests are displayed which were used to test for significant differences in the relative abundances (%) of microbial groups between inoculated and non-inoculated seedlings.

Microbial group	Non-inoculated	Inoculated	Significance
Total PLFA (nmol/g)	481.0 ± 16.6	451.0 ± 16.5	T= 1.29, p-value = 0.21
Fungi : Bacteria ratio	0.21 ± 0.01	0.19 ± 0.00	T= 2.40, p-value = 0.02
Total bacteria (%)	81.78 ± 0.40	83.01 ± 0.29	T= 2.48, p-value = 0.02
Total fungi (%)	17.56 ± 0.40	16.39 ± 0.30	T= 2.35, p-value = 0.03
Gram Negative (%)	43.08 ± 0.22	43.32 ± 0.28	T= 0.69, p-value = 0.49
Gram Positive (%)	18.20 ± 0.37	18.32 ± 0.29	T= 0.26, p-value = 0.80
Other Eubacteria (%)	15.77 ± 0.22	17.26 ± 0.23	T= 4.73, p-value < 0.001
Saprophytic fungi (%)	10.72 ± 0.21	10.43 ± 0.16	T= 1.09, p-value = 0.28
Actinomycetes (%)	4.74 ± 0.14	2.41 ± 0.09	T= 3.65, p-value < 0.001
AM Fungi (%)	3.81 ± 0.14	3.55 ± 0.14	T= 1.33, p-value = 0.19
EM Fungi (%)	3.03 ± 0.15	2.41 ± 0.09	T= 3.49, p-value = 0.002

5.5 Discussion

5.5.1 Pronounced shifts in soil bacterial communities following seedling infection

Pronounced and significant differences were found in soil bacterial community composition between inoculated and non-inoculated seedlings, which may be explained by changes in the availability of soil nutrients and labile carbon (C) following seedling disease expression. Kauri seedlings inoculated with *P. agathidicida* experienced significant losses in their root mass, a factor which may have reduced their release of root exudates. Plant root exudates provide important inputs of easily available, carbon rich compounds that help to sustain soil microbial activity (De Graaff et al., 2010). Inoculated seedlings may have experienced an increased abundance of soil bacteria taxa with oligotrophic nutritional strategies which are less dependent on a rich supply of labile C, compared to copiotrophic bacteria which prefer nutrient rich soils with larger pools of labile C (Fierer et al., 2007). The Firmicutes, including the genera *Bacillus* and *Paenibacillus*, had a significantly higher relative abundance in soils from inoculated seedlings. Although the Firmicutes have yet to be assigned a definitive oligotrophic or copiotrophic nutritional strategy (Fierer et al., 2007), they are commonly abundant in low nutrient soils due to their ability to use more recalcitrant carbon and inorganic nutrients (Llado et al., 2017). For example, *Bacillus* and *Paenibacillus* have demonstrated the ability to degrade phenolic compounds (Llado et al., 2017; Tian et al., 2014) which are found in high concentrations in kauri leaf litter (Verkaik et al., 2006; Wyse et al., 2014). The increased abundance of these bacterial taxa in soils from inoculated seedlings may reflect their competitive advantage in the soil environment following seedling disease expression which is driving down the abundance of other bacterial taxa.

The Actinomycetales had a significantly lower relative biomass and OTU abundance in soils from inoculated seedlings. This contrasts to the findings of **Chapter 4** which found a higher OTU abundance of Actinobacteria in symptomatic kauri soils. The Actinomycetales are considered putative copiotrophs which play key roles in the degradation of soil organic matter (Ding et al., 2015; Fierer et al., 2012; Navarrete et al., 2015). Therefore, their decreased abundance in soils from inoculated seedlings may be a response to changes in soil nutrient availability and C supply following seedling disease expression. This is supported by the findings of Yao et al. (2014), who observed *Escherichia coli* infested soils to have a decreased biomass of the Actinomycetes. These declines were attributed to *E. coli* infested soils shifting from a copiotrophic to an oligotrophic environment which promoted a higher activity of more well-adapted and competitive bacteria. In contrast, the increased abundance of Actinomycetales in symptomatic kauri soils (**Chapter 4**) may be a response to the larger inputs of dead plant tissue in these soils following tree dieback.

5.5.2 Significant reductions in fungal: bacterial ratio in soils from inoculated seedlings

Soils associated with inoculated seedlings had a significantly lower fungal: bacterial biomass ratio, which may be driven by their increased bacterial diversity and biomass. As discussed previously, the shifts in soil bacterial community composition in inoculated seedlings may suggest that select bacterial taxa have a competitive advantage in the soil environment following seedling disease expression. During tree decay, soil bacteria are often the initial colonisers of dead plant tissues due to their ability to utilise readily available sugars (Greaves, 1971). Furthermore, the changes which occur to fungal communities following plant decay are linked to their ability to compete and utilise the available resources (Prewitt et al., 2014). Therefore, the higher relative abundance of soil bacteria in inoculated seedlings may suggest that they are better adapted than soil fungi at utilising the available substrates. In addition, the significant decreases in the soil fungal: bacterial ratio of inoculated kauri seedlings may be driven in part by their reduced biomass of the Actinomycetes. This is because the Actinomycetes play key roles in supporting soil fungal communities through their functional roles in C turnover and availability (Ma et al., 2013; Yao et al., 2014).

One other factor which may have contributed to the declines in soil fungal biomass in inoculated seedlings is their increased abundance of *P. agathidicida*. *Phytophthora* pathogens are hemibiotrophic pathogens, meaning they have an initial stage of parasitizing living host cells before continuing to live off dead host tissue as saprophytes (Horbach et al., 2011; Thines, 2013). A previous study by Bellgard et al. (2016) observed an increased abundance of *P. agathidicida* within and outside the root zone of infected kauri. These findings are supported by this present study, as all cDNA extracts of soils collected from around the roots of inoculated seedlings tested positive for *P. agathidicida*. The increased abundance of *P. agathidicida* in soils from inoculated seedlings may have competitively excluded resident fungal communities for space and nutrients at the root zone, thus contributing to their decline in biomass. Root exudates released by plants allow plant rhizospheres to support the activities of soil microorganisms (Baldrian, 2016). However when soil pathogens invade new environments they compete with resident microorganisms for space and nutrients (van Elsas et al., 2012). Compared to the symptomatic kauri studied in **Chapter 4**, the smaller root area of kauri seedlings may have increased the rates of resource competition and limited activities of the resident soil fungal communities. As a control variable, this study inoculated 'non-inoculated' kauri seedlings with blank V8 agar plugs. However future studies may wish to inoculate control seedlings with a non-pathogenic *Phytophthora* species so that both inoculated and control seedlings are subject to similarly increased rates of resource competition. This form of control could not be used in this study as our current understanding on the range of *Phytophthora*

species that are pathogenic against kauri is inconclusive. Despite *P. agathidicida* being identified as the primary causal agent of kauri dieback, several other *Phytophthora* species have also been linked to kauri ill health (Horner & Hough, 2014; Waipara et al., 2013).

5.5.3 The taxonomic responses of soil bacterial communities to seedling infection

Several bacterial genera belonging to the Firmicutes (e.g. *Clostridium, Bacillus* and *Paenibacillus*) and the Burkholderiales were found in significantly higher abundance in soils from inoculated seedlings. Members of the Firmicutes have been previously found common to disease suppressive soils (Mendes et al., 2011; Xiong et al., 2017) and strains of *Bacillus* and *Paenibacillus* have been developed for use as biocontrol agents (Govindasamy et al., 2010; Kim et al., 2009; Ryu et al., 2003). For example, *Bacillus* has been identified to produce anti-fungal compounds that are suppressive against *Phytophthora nicotianae*, such as bacillomycin and surfactin (Ros et al., 2017). Several studies have identified *Paenibacillus* and *Bacillus* strains to antagonise a variety of *Phytophthora* species, such as *P. capsica* (Akgül & Mirik, 2008; Jung et al., 2004), *P. cactorum* (Bae et al., 2004; Utkhede, 1984), *P. palmivora* (Timmusk et al., 2009), *P. nicotianae* (Ren et al., 2012; Wu et al., 2018) and *P. infestans* (Caulier et al., 2018). Several *Burkholderia* species have been identified to produce broad spectrum antibiotics and volatile organic compounds (VOCs) which can antagonise plant pathogens (Schmidt et al., 2009; Tenorio-Salgado et al., 2013). Benítez and Gardener (2009) identified members of the Burkholderiales that significantly inhibited the growth and development of a range of plant pathogens, including *Phytophthora sojae*. Furthermore, *B. cepacia* was identified as an effective biocontrol agent against *P. capsici*, the causal agent of late blight in pepper (Sophearetth et al., 2013).

Disease suppression can be induced into soils following disease outbreak due to the selection and enrichment of microorganisms which possess functional traits suppressive against pathogens (Baker & Cook, 1974; Chapelle et al., 2016; Raaijmakers & Mazzola, 2016; Weller et al., 2002). The suppressive soil memory which can develop after disease outbreak has been applied to develop synthetic soil bacterial communities that can significantly reduce plant disease expression (Bakker et al., 2018; Berendsen et al., 2018). For example, Berendsen et al. (2018) identified that downy mildew infection of *Arabidopsis thaliana* promoted the abundance of the bacterial genera *Microbacterium*, *Stenotrophomonas*, and *Xanthomonas*. Co-inoculations of these bacteria were able to induce plant systemic resistance against downy mildew upon further infection. Such findings by Berendsen et al. (2018) offer promising insight into how soil disease suppression can be promoted by enriching the population levels of microbial taxa which exhibit strong responses to plant infection. To extend the findings of this kauri seedling study, the bacterial taxa that showed a strong response to seedling

infection could be amended in soils to a higher population density. Kauri seedlings grown in these amended soils could then be infected with *P. agathidicida* to identify if the increased abundance of the bacterial taxa prevented or reduced plant disease expression.

5.5.4 The taxonomic responses of soil fungal communities to seedling infection

There were only small differences in the composition of soil fungal communities between inoculated and non-inoculated seedlings. Moreover, the previously reported literature on the disease suppressive properties of the fungal taxa found significantly higher in soils from inoculated seedlings (e.g. Hyaloscyphaceae and *Melinomyces*) is limited. For example, although members of the Hyaloscyphaceae have been reported to function as saprophytes and root endophytes (Kernaghan & Patriquin, 2011; Nakamura et al., 2018; Quijada et al., 2015), little has been reported on their roles in plant protection and pathogen defence. Narisawa et al. (2004) reported *Melinomyces variabilis* to be a potential biocontrol agent against *Verticillium* and *Fusarium* wilt. However, further research found that root colonisation rates of *M. variabilis* in non-ericaceous plant species to be low, with successful root colonisation being required to protect plants against soil pathogens (Ohtaka & Narisawa, 2008; Vohník et al., 2013). Furthermore, the *Melinomyces* have a wide functional variability and have been described as dark septate endophytes, ectomycorrhizae or ericoid mycorrhizae depending on the host species under study (Grünig et al., 2011; Kernaghan & Patriquin, 2011). For the purposes of this study, more targeted research is needed to assess the potential roles of these fungal taxa in supporting kauri health upon pathogen attack.

5.5.5 Contrasts with previous kauri dieback research

The results of this study found a stronger response from soil bacterial communities compared to soil fungal communities to the inoculation of kauri seedlings with *P. agathidicida*. In contrast, **Chapter 4** identified that symptomatic kauri had a significantly higher soil fungal diversity compared to asymptomatic kauri, alongside more pronounced shifts in soil fungal community composition between asymptomatic and symptomatic kauri. The differences in findings between this study and **Chapter 4** may be a result of the major differences in the environmental characteristics of the two plant systems under study. The experimental conditions of this kauri seedling infection study meant that soil microbial communities were studied using a closed container pot trial in association with younger, smaller seedlings. When studying the role of soil microorganisms in responding to forest disease outbreaks, it is important to acknowledge how they function within their ecosystems (Baldrian, 2016). For ecologically unique habitats such as kauri forests, properties of the soil microbiota are shaped by the influences that dominant tree species exert on their surrounding soil environment (Wyse et al., 2014). The conditions imposed during the seedling experiment may not

have elicited the same functional response of the soil fungal communities to tree attack as would have occurred when within the influence of their original kauri host. The increased fungal diversity observed in symptomatic kauri soils **(Chapter 4)** was attributed to increased outputs of dead plant tissue following tree dieback. However, for this seeding experiment, disease expression did not produce such large outputs of dead plant tissue due to the smaller root, shoot and leaf area of each seedling. Plant litter contributes to the majority of organic matter inputs in forest soils and can support proliferous populations of saprophytic fungi (Baldrian, 2016). The comparatively limited plant litter outputs following seedling mortality could not support large increases in secondary saprotrophic fungi as observed in **Chapter 4**. In addition, the influx of secondary colonising fungi was restricted as it was a closed container experiment which allowed for no large fluctuations of fungal populations in response to tree disease expression.

The relatively short six-week inoculation period that was selected before characterising the response of the soil microbiota to seedling infection may have contributed to the limited response of soil fungal communities. Soil bacteria are often the initial colonisers of dead plant tissue and it is not until the later stages of plant decay that populations of soil fungi rise as they are better able to utilise partially degraded plant tissues (Greaves, 1971; Prewitt et al., 2014). To build upon the findings of this seedling infection study, future research may aim to study the response of the soil microbiota over longer time periods (i.e. 3 to 6 months) and at multiple time points throughout the inoculation period. This would allow us to study the successional changes in soil microbial communities over time, which may provide more information about the response of soil fungal communities to kauri infection.

5.6 Conclusion

This study aimed to observe the short-term responses of the soil microbiota six weeks following the infection of kauri seedlings with *P. agathidicida*. Seedlings inoculated with *P. agathidicida* experienced pronounced and rapid shifts in soil bacterial community composition. The bacterial taxa found in significantly higher relative abundance in soils from inoculated seedlings have previously reported roles in plant disease suppression. Before we can begin assessing the potential of these taxa to function as biocontrol agents against *P. agathidicida*, their interaction with *P. agathidicida* needs to be studied in a more targeted manner. Soil fungal communities associated with kauri seedlings inoculated with *P. agathidicida* showed only minor responses to seedling infection. This contrasts to **Chapter 4** which observed a strong response of soil fungal communities to the expression of kauri dieback in mature kauri. The results of this kauri seedling infection study identified members of the soil microbiota that were the primary respondents to plant infection.

However, to better understand the changes in microbial community dynamics following seedling infection, particularly for soil fungal communities, future studies should also to be performed which study the response of the soil microbiota over a longer time period.

5.7 Acknowledgements

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Chapter 6

Identifying *Burkholderia* and *Penicillium* strains isolated from kauri (*Agathis australis*) soils that inhibit the mycelial growth of *Phytophthora agathidicida*.

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Supplementary material for this chapter is provided in Appendix D.

6.1 Abstract

Phytophthora agathidicida is a highly virulent pathogen of kauri (*Agathis australis*) and the primary causal agent of dieback disease in New Zealand's kauri forests. This study aimed to identify microbial strains isolated from kauri forest soils that inhibited the growth of *P. agathidicida in vitro*. Three different forms of bioassays were used to assess the inhibition of each microbial strain against the mycelial growth of *P. agathidicida*. Furthermore, solid phase micro extraction coupled with gas chromatography mass spectrometry (SPME/GCMS) was performed on the head space (HS) of each microbial strain to identify if they emitted inhibitory volatile organic compounds (VOCs). This research identified several bacterial strains belonging to the genus *Burkholderia* that inhibited the mycelial growth of *P. agathidicida.* Furthermore, several VOCs produced by these strains have been identified which may be responsible for inhibition. Several strains of *Penicillium* were also found to inhibit *P. agathidicida*, with the culture filtrates of one strain being found to strongly inhibit *P. agathidicida* mycelial growth. The strains of *Burkholderia* and *Penicillium* isolated in this study appear to exhibit multiple modes of antagonism against *P. agathidicida*, including microbial competition and the production of diffusible and volatile anti-microbial compounds. Although further research is needed to define their mechanisms of inhibition, these findings have identified candidate microbial antagonists of *P. agathidicida* which could potentially be used for the management of kauri dieback.

6.2 Introduction

The spread of kauri dieback, caused by the pathogen *Phytophthora agathidicida*, poses a serious threat to the long term health of New Zealand's kauri (*Agathis australis*) forests (Beever et al., 2009). Kauri dieback was discovered to be afflicting kauri on Great Barrier Island (Hauraki Gulf, Auckland) in the 1970s and is now widespread across the majority of mainland kauri forests (Gadgil, 1974; Waipara et al., 2013). Kauri dieback is a root and collar rot disease that is spread through the movement of soil and root pieces infested with *P. agathidicida* (Bellgard et al., 2016). Current management strategies primarily focus on disease containment by preventing further spread of *P. agathidicida* infested soils into new forested areas (Bradshaw et al., 2020). Several research efforts have been made to manage the disease which include tree phosphite injections (Horner & Hough, 2013), oospore deactivation (Dick & Kimberley, 2012), screening kauri for genetic resistance (Herewini et al., 2018) and mātauranga Māori guided discovery of native plant bio-actives with anti-*Phytophthora* properties (Lawrence et al., 2019).

Despite this, there has been limited research on how the soil microbiota may suppress kauri dieback by supporting tree health or directly antagonising *P. agathidicida*. Several plant diseases caused by *Phytophthora* pathogens have been managed using chemical control, such as potato (*Solanum tuberosum*) late blight caused by *P. infestans* (Majeed et al., 2017) and pepper (*Capsicum annuum*) blight caused by *P. capsici* (Kim et al., 2010). However, there are concerns over the widespread use of chemical control due to the associated environmental impacts and the development of fungicide resistance (O'Brien, 2017). Disease management strategies that integrate the use of microbial agents to antagonise pathogens and prevent plant infection are considered a more sustainable, less environmentally impactful alternative (Bhusal & Mmbaga, 2020). For the management of soil-borne plant pathogens, it is preferential to target the discovery of microbial antagonists to the host associated soil microbiota. More often, members of the resident soil microbiota are more likely to establish, proliferate and elicit the functional traits required to suppress plant disease when applied to soils in the field (Raaijmakers et al., 2009; Zohara et al., 2016).

Chapter 4 identified significant differences in the composition of soil fungal and bacterial communities between asymptomatic and symptomatic kauri trees in Waipoua Forest (Northland Region, New Zealand). Furthermore, several of the microbial genera found in significantly higher relative abundance in asymptomatic kauri soils have been previously reported to inhibit various other *Phytophthora* pathogens. These include genera such as *Penicillium* (Fang & Tsao, 1995; Ma et al., 2008), *Trichoderma* (Ahmed et al., 2000; Bae et al., 2016; Widmer, 2014) and *Pseudomonas* (Caulier et al., 2018; Tran et al., 2007; Zohara et al., 2016). Although **Chapter 4** identified microbial

taxa associated with potentially disease suppressive soils (i.e. asymptomatic host states), it did not assess whether these taxa share an antagonistic interaction with *P. agathidicida*. Therefore, additional research is needed to isolate and identify members of the kauri soil microbiota that can be demonstrated to inhibit the growth of *P. agathidicida*.

This study aimed to isolate microbial strains from kauri forest soils and identify those which suppressed the mycelial growth of *P. agathidicida*. Three different bioassay types were used to assess each microbial strain: dual culture bioassays, culture filtrate bioassays and split plate bioassays. Dual culture bioassays were selected as the primary form of bioassay because they provided a fast and easily reproducible way of screening a large number of microbial strains for their potential to inhibit *P. agathidicida* (Kunova et al., 2016; Zohara et al., 2016). To provide more insight into the mechanisms driving mycelial inhibition, culture filtrate bioassays were used to assess whether diffusible compounds (i.e. metabolites) produced by the microbial strains were contributing to inhibition (Ma et al., 2008; Zohara et al., 2016). In addition, split plate bioassays were used to assess whether the microbial strains produced volatile organic compounds (VOCs) that may be associated with the inhibition of *P. agathidicida* (Syed-Ab-Rahman et al., 2019).

Volatile organic compounds (VOCs) are secondary metabolites released by soil microorganisms. Some microbial VOCs are known to exhibit anti-microbial properties against a wide range of plant pathogens (de Boer et al., 2019). Microbial VOCs commonly belong to the chemical classes of alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids and esters (Schmidt et al., 2015). They are small lipophilic compounds with a low molecular mass, a high vapour pressure and a low boiling point (Piechulla & Degenhardt, 2014). These properties allow VOCs to readily evaporate and diffuse through water and gas filled pores in the soil. When compared to diffusible organic compounds, VOCs can interact with and influence the activities of other microorganisms over larger distances and when physically separated within the soil (Bitas et al., 2013; Schmidt et al., 2015). VOCs play a significant role in antagonising pathogens and have been demonstrated to inhibit several *Phytophthora* pathogens including *P. infestans* (Elsherbiny et al., 2020; Hunziker et al., 2015; Kumar et al., 2018), *P. capsici* (Syed-Ab-Rahman et al., 2019) and *P. cinnamomi* (Méndez-Bravo et al., 2018). This study used solid phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS) to extract and identify the VOCs from the head space (HS) of the fungal and bacterial strains shown to inhibit *P. agathidicida.* HS-SPME/GCMS analysis was used to accompany the results of the split plate bioassays so that the potential mechanisms driving the mycelial inhibition of *P. agathidicida* could be explored.

6.3 Methods

6.3.1 Isolation of microbial strains from kauri soil

The microbial strains screened against *P. agathidicida* during this study were isolated from organic layer soils that were collected from asymptomatic kauri trees across Waipoua Forest (Northland Region, New Zealand). Soil samples were collected during the previous soil sampling rounds outlined in **Chapter 4** (**Section 4.3.1**) and **Chapter 5** (**Section 5.3.1**) and were stored at 4°C in darkness prior to use in this study. Only kauri soil samples that tested negative for presence of *P. agathidicida* when screened using a soil baiting bioassay and real-time PCR assay (Supplementary Methods B.1, Appendix B) were used in this study.

Microbial strains were isolated from kauri soils using soil serial dilutions and selective agar plating (Zohara et al., 2016). Eight soil samples were randomly selected from the store of uninfected kauri soil described above. Per sample, triplicate 1 g subsamples were suspended in 100 ml of sterile water. Soil suspensions were shaken at 140 rpm on an orbital shaker (Ratek Instruments Pty Ltd., Australia) for 4 hours at room temperature before being left to settle on the laboratory benchtop for 1 hour. Once settled, 1 ml of each soil suspension was diluted at a 1: 10 concentration with sterile water. Following this, 1 ml of each serial dilution was plated onto a bacterial selective and a fungal selective agar plate and spread evenly across the surface of the plate using a cell spreader. Recipes for the bacterial selective and fungal selective agar plates are listed in Supplementary Methods D.1, Appendix D.

Selective agar plates were incubated for up to 4 days in darkness at 21.5° C, which is the optimum growth temperature of *P. agathidicida* (Weir et al., 2015). This incubation temperature was selected to cultivate microbial strains compatible for growth in bioassay screenings with *P. agathidicida*. Plates were inspected every 24 hours, with emerging fungal and bacterial colonies immediately subcultured onto potato dextrose agar (PDA) (Oxoid Ltd., UK) and nutrient agar (NA) (Oxoid Ltd., UK), respectively. For each selective agar plate, emerging colonies which had visually different colony morphologies were those selected for sub culturing. By the end of this selective isolation process, 164 bacterial strains and 170 fungal strains were isolated from kauri soils and put forward for pre-screening. Microbial strains were temporarily stored on half strength PDA agar or NA plates at in darkness at 4°C prior to being prepared for use in the bioassays.

6.3.2 *P. agathidicida* source material

The *P. agathidicida* strain NZFS3770 was used to conduct experiments throughout the duration of this study. This strain was recieved from the National Forest Culture Collection (Scion, Rotorua, New

Zealand) and was originally isolated in 2006 from the Coromandel, New Zealand. The full genome of *P. agathidicida* NZFS3770 was sequenced by Studholme et al. (2016) and is deposited in GenBank under the accession number LGTS00000000. This strain was routinely cultured on CRNH medium (Herewini et al., 2018) to preserve pure cultures and then maintained on 20% clarified V8 agar in darkness at 21.5°C prior to use in experiments (Lawrence et al., 2017).

6.3.3 Pre-screening of microbial strains

Dual culture bioassays were selected as the primary bioassay type to screen all the 164 bacterial strains and 170 fungal strains against *P. agathidicida*. Due to the number of strains that required screening, pre-screening rounds were conducted prior to beginning more detailed bioassays. During pre-screenings, dual culture bioassays (methods outlined below in **Section 6.3.4**) were prepared in triplicate for each microbial strain and only strains that reduced the mycelial growth (mm) of *P. agathidicida* when compared to control were retained for further bioassay analysis. Only the results of the microbial strains which passed pre-screening are presented in this chapter.

6.3.4 Dual culture bioassays

Microbial strains that passed pre-screening were screened against *P. agathidicida* again using dual culture bioassays. To prepare bacterial strains for the dual culture bioassays, each bacterial strain was streaked out onto an NA plate and incubated in darkness at 21.5°C for 24 hours to produce single colonies. Single colonies were inoculated into 15 ml of sterile tryptone soya broth (TSB) (Oxoid Ltd., UK) using a 1µl inoculation loop. Broth cultures were shaken at 100 rpm on an orbital shaker (Ratek Instruments Pty Ltd., Australia) in darkness at 21.5°C for 48 hours. After incubation, broth cultures were centrifuged (4000 rpm for 20 minutes) and the supernatant was discarded. Bacterial pellets were suspended in 500 µl of sterile water. To prepare both the fungal strains and *P. agathidicida* for dual culture bioassays, a 5mm diameter agar plug of each strain was subcultured onto a fresh PDA plate and incubated in darkness at 21.5°C for 5 days.

To set up the dual culture bioassays, a 5mm diameter agar plug of *P. agathidicida* was taken from the leading mycelial edge and placed into the centre of a PDA plate. For each fungal strain, two 2.5 mm diameter agar plugs were positioned 2 cm away from the *P. agathidicida* agar plug and on opposite sides of the PDA plate. For bacterial strains, PDA plates were inoculated with two 50 µl doses of liquid culture in the same positions as described for fungal strains. Control plates were prepared by inoculating PDA plates that contained a 5mm diameter agar plug of *P. agathidicida* with either two 2.5 mm diameter blank PDA agar plugs or two 50 µl doses of sterile water. Dual culture bioassays were incubated in darkness at 21.5°C for 1 week before *P. agathidicida* was measured for

its mycelial growth (mm). Dual culture bioassays were repeated three times and for each of the three rounds, five replicates were performed for each microbial strain.

6.3.5 Culture filtrate bioassays

Prior to setting up the culture filtrate bioassays, broth cultures of each bacterial strain were prepared as described in **Section 6.2.2**. Following incubation at 21.5^{o}C for 48 hours, bacterial cultures were centrifuged (4000 rpm for 40 minutes) and the resulting supernatant was double filtered through cellulose acetate ReliaPrep™ 0.2 µM syringe filters (Ahlstrom-Munksjö, Finland) to a obtain a cell free filtrate. Methods for the fungal strains followed those described above for bacterial strains. However fungal broth cultures were prepared by inoculating 25 ml of sterile malt extract broth (MEB) (Oxoid Ltd., UK) with three 5 mm diameter agar plugs of each fungal strain which were then incubated in darkness at 25^{o}C for 5 days.

To set up the culture filtrate bioassays, one 5 mm diameter agar plug of *P. agathidicida* was subcultured onto a PDA plate. Following this, 500 µl of bacterial/fungal cell free filtrate was pipetted directly onto the *P. agathidicida* agar plug (Ma et al., 2008; Vinale et al., 2006; Zohara et al., 2016). Control plates consisted of one 5 mm diameter agar plug of *P. agathidicida* which was inoculated with either 500 µl of sterile TSB or MEB. Culture filtrate bioassays were incubated in darkness at 21.5^{o}C for 1 week before *P. agathidicida* was measured for its mycelial growth (mm). Culture filtrate bioassays were repeated three times and for each of the three rounds, five replicates were performed for each microbial strain.

6.3.6 Split plate bioassays

Methods for split plate bioassays were modified based methods outlined by Syed-Ab-Rahman et al. (2019). Split plate petri dishes (Thermo Fisher Scientific, New Zealand) which had a 0.5 mm wide strip of agar cut out to further separate each microbial strain from *P. agathidicida* were used to set up the bioassays. Bacterial broth cultures and fungal agar cultures were prepared as described in **Section 6.2.2**. Following preparation, either 100 µl of bacteria or a 5 mm diameter agar plug of fungi was inoculated onto one side of a split PDA plate. The opposite side of the split PDA plate was then inoculated with a 5 mm diameter agar plug of *P. agathidicida*. Plates were sealed with Parafilm M® (Amcor, Switzerland) and incubated in darkness at 21.5^{o}C for 7 days before *P. agathidicida* was measured for its mycelial growth (mm). Split plate bioassays were repeated three times and for each of the three rounds, five replicates were performed for each microbial strain.

6.3.7 Calculation of *P. agathidicida* inhibition

The mycelial inhibition (MI) value (%) of each bioassay plate was calculated using the formula:

*'((**C** - **A**) / **C**) x 100'*

Where, **C** is the growth (mm) of *P. agathidicida* on control plates and **A** is the growth (mm) of *P. agathidicida* on plates inoculated with each microbial strain. Significant differences in MI values (%) between the experimental and control dual culture bioassays were determined using Student's T tests.

6.3.8 Profiling of VOCs using HS-SPME/GCMS

Head space solid-phase micro extraction coupled with gas chromatography-mass spectrometry (HS-SPME/GCMS) was performed to profile the VOCs released by the microbial strains. Each fungal strain was subcultured onto a PDA plate and incubated at 21.5^{o}C in darkness for 7 days. Following this, four 5 mm diameter agar plugs were transferred into a 20 ml amber glass head space vial (Supelco Analytical, Sigma Aldrich) and incubated at 21.5^{o}C for 24 hours. Liquid cultures of each bacterial strain were prepared as described in **Section 6.2.2**. Following preparation, 100 µl of bacteria was inoculated into a head space vial containing 3 ml of NA and incubated at 21.5^{o}C for 24 hours. To prepare *P. agathidicida* only control vials, an agar plug of *P. agathidicida* was freshly subcultured onto PDA and incubated in darkness at 21.5^{o}C for 5 days. Following incubation, four 5 mm diameter agar plugs were transferred into a head space vial and incubated at 21.5^{o}C for 24 hours. As further controls, head space vials containing either four 5 mm diameter blank PDA plugs or 3ml of NA inoculated with sterile water were prepared and incubated in the same conditions as the other sample vials. Three replicate head space vials were set up for all experimental and control samples analysed. During incubation, head space vials were plugged with a sterile cotton ball to maintain aerobic conditions. Following incubation, cotton ball plugs were discarded, and head space vials were sealed with an 18 mm thread magnetic screw cap that contained a 1.5 mm thick blue PTFE/silicone septum (Supelco Analytical, Sigma Aldrich).

Methods for the HS-SPME/ GCMS analysis followed those outlined by Nieto-Jacobo et al. (2017) and Stoppacher et al. (2010). A Shimadzu QP2010 gas chromatograph-mass spectrometer (Shimadzu Corporation, Japan) fitted with a CTC-CombiPAL XYZ auto sampler and a Restek Rxi-5 ms fused silica capillary column (HP5-MS 30 m × 0.25 mm × 0.25 µm, Bellefonte, PA, USA) was used to perform the analysis. VOCs were extracted from the sample head space vials for 30 minutes without agitation using an SPME fibre that had a 65 µM polydimethylsiloxane/ divinylbenzene (PDMS/DVB) coating.

After injection, volatiles bound to the fibre were desorbed for 2 minutes in a split/split less injector at 250°C. The oven temperature programme was held at 40°C for 2 minutes; raised 10°C per minute to 200°C; further raised 25°C per minute to 260°C and then held at 260°C for 5 minutes. Helium was used as carrier gas at a constant flow rate of 1 ml per minute.

The acquisition and processing software GCMS solution version 4.45 (Shimadzu Corporation, Japan) was used to identify the best match for each detected compound. This software used the NIST 2011 and Wiley 10 mass spectral libraries to identify compounds in conjunction with their reported linear retention index (LRI) information. An alkane standard solution C_8-C_{20} (Sigma-Aldrich, Australia) was run to calculate LRI values for each identified compound. The reported LRI values for identified compounds were compared to previously reported literature LRI values, obtained from ChemSpider- http://www.chemspider.com/ and NIST Chemistry WebBook- https://webbook.nist.gov/chemistry/ (Table D.4, Appendix D).

Only compounds with an LRI value within ± 5 % of their literature LRI value were accepted as an identified substance. Furthermore, only compounds with a compound match rate of over 85% and that were present in at least two of the three biological replicates were retained as accepted compounds (Table D.3, Appendix D). Compounds detected in control head space vials were designated as background noise and removed as identified compounds detected in sample head space vials. Following identification, individual VOCs were assigned into chemical classes (i.e. monoterpenoid, alkene, benzenoid) using mVOC 2.0 (Lemfack et al., 2018) and ClassyFire (Djoumbou Feunang et al., 2016).

6.3.9 Genomic identification of microbial isolates

Only microbial strains that passed pre-screening were identified using Sanger sequencing of their 16S rRNA (for bacteria) or ITS (for fungi) gene region. Microbial DNA was extracted from liquid bacterial cultures and fungal agar plugs using a DNeasy UltraClean Microbial Kit (Qiagen, Germany) according to manufacturer's instructions. The quantity (ng) and quality (A260: A280 ratio) of DNA extracts were determined using a Nanodrop® Spectrophotometer. Bacterial DNA extracts were diluted at a 1:10 concentration prior to PCR amplification.

The 16S rRNA gene region was amplified using the forward and reverse primers 341F/785R (Thijs et al., 2017) and the ITS2 gene region was amplified using the forward and reverse primers ITS3/ITS4 (Op De Beeck et al., 2014). Each 25 µl PCR reaction consisted of 0.5 µl of forward and reverse primers at a 10 µM concentration; 4 µl HOT FIREPol Blend Master Mix (Solis Biodyne, Estonia); 19 µl of nuclease free water (Invitrogen, Massachusetts USA) and 1 µl of DNA. PCR thermocycling

conditions were 95°C for 12 minutes, followed by 30 cycles of 95°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final extension phase of 75°C for 5 minutes. PCR products were ran on 1% agarose gel at 100 V for 15 minutes to confirm amplification of the desired band. PCR products were cleaned using a Magnetic Bead PCR Cleanup Kit (Geneaid Biotech Ltd., Taiwan) and sequenced using Sanger sequencing at the Lincoln University DNA Sequencing facility (Lincoln, New Zealand). Following sequencing, low quality ends were trimmed and forward and reverse reads were aligned using UniPro UGENE (version 36.0) software (Okonechnikov et al., 2012). Microbial strains were identified to genus level based on the identity of their closest match from the NCBI BLAST rRNA and ITS database (GenBank). Bacterial 16S rRNA and fungal ITS2 gene regions were deposited in the NCBI database under the GenBank accession numbers shown in Table 6.1.

6.4 Results

6.4.1 Genomic identification of strains

From the 11 bacterial strains identified to significantly inhibit *P. agathidicida* mycelial growth in dual culture bioassays, nine were identified as belonging to the genus *Burkholderia*, one was identified as a *Paraburkholderia*, and one as *Pseudomonas*. All nine of the fungal strains that significantly inhibited *P. agathidicida* in dual culture bioassays were identified to belong to the genus *Penicillium* (Table 6.1). Images of each fungal and bacterial strain can be seen in Figure D.1 and D.2, Appendix D.

Table 6.1. The taxonomic assignment of each bacterial and fungal strain was performed to genus level based off the identity of their closest match when their 16S rRNA/ ITS2 gene region sequences were searched in the NCBI BLAST database.

Strain ID	Accession number	Closest match	Expect value	% match
Burkholderia strain ks20_b4	MW041148	*B. catarinensis* (NR_153664.1)	0.0	99.15
Burkholderia strain ks20_b8	MW040830	*B. catarinensis* (NR_153664.1)	0.0	98.44
Paraburkholderia strain ks20_b72	MW040841	*P. metalliresistens* (NR_118054)	0.0	97.51
Burkholderia strain ks20_b9	MW040831	*B. catarinensis* (NR_153664.1)	0.0	97.87
Burkholderia strain ks20_b69	MW041147	*B. catarinensis* (NR_153664.1)	0.0	97.62
Burkholderia strain ks20_b71	MW040834	*B. catarinensis* (NR_153664.1)	0.0	99.13
Burkholderia strain ks20_b31	MW040833	*B. catarinensis* (NR_153664.1)	0.0	98.50
Burkholderia strain ks20_b16	MW041149	*B. catarinensis* (NR_153664.1)	0.0	99.15
Burkholderia strain ks20_b74	MW040835	*B. catarinensis* (NR_153664.1)	0.0	98.91
Burkholderia strain ks20_b12	MW040832	*B. catarinensis* (NR_153664.1)	0.0	98.54
Pseudomonas strain ks20_b65	MW040836	*P. helleri* (NR_148763.1)	0.0	97.86
Penicillium strain ks20_ f10	MW040805	*P. thomii* (NR_077159.1)	2e-173	99.11
Penicillium strain ks20_ f20	MW040810	*P. bialowiezense* (NR_165994.1)	1e-165	96.58
Penicillium strain ks20_ f52	MW040812	*P. montanense* (NR_138270.1)	2e-163	98.76
Penicillium strain ks20_ f54	MW040813	*P. thomii* (NR_077159.1)	8e-167	97.66
Penicillium strain ks20_ f14	MW040806	*P. daejeonium* (NR_158791.1)	1e-169	98.23
Penicillium strain ks20_ f15	MW040807	*P. kiamaense* (NR_137899)	2e-168	99.69
Penicillium strain ks20_ f18	MW040808	*P. malachiteum* (NR_120271.1)	0.0	99.15
Penicillium strain ks20_ f19	MW040809	*P. montanense* (NR_138270.1)	5e-174	98.55
Penicillium strain ks20_ f30	MW040811	*P. montanense* (NR_138270.1)	1e-175	98.29

6.4.2 *In vitro* bioassays

Dual culture bioassays

When tested using dual culture bioassays, all of the fungal and bacterial strains that passed pre-screening (Table 6.1) significantly reduced *P. agathidicida* mycelial growth compared to *P. agathidicida* only control plates (p-value < 0.001, Figure 6.4 and Figure 6.5).

Fungal strains with the highest mean % mycelial inhibition (MI) values were *Penicillium* strain ks20_f18 (58.28 ± 1.55), *Penicillium* strain ks20_f30 (57.27 ± 1.20) and *Penicillium* strain ks20_f52 (55.27 ± 1.26). Bacterial strains with the highest mean % MI values were *Burkholderia* strain ks20_b71 (60.88 ± 3.22), *Burkholderia* strain ks20_b12 (59.91 ± 1.84) and *Burkholderia* strain ks20_b69 (59.78 ± 1.37). Images of fungal and bacterial dual culture bioassays can be seen in Figure 6.1 and Figure 6.2. The MI values (mean ± SE) of the dual culture bioassays and the results of the Student's T tests are shown in Tables D.1 for (fungal strains) and D.2 (bacterial strains), Appendix D.

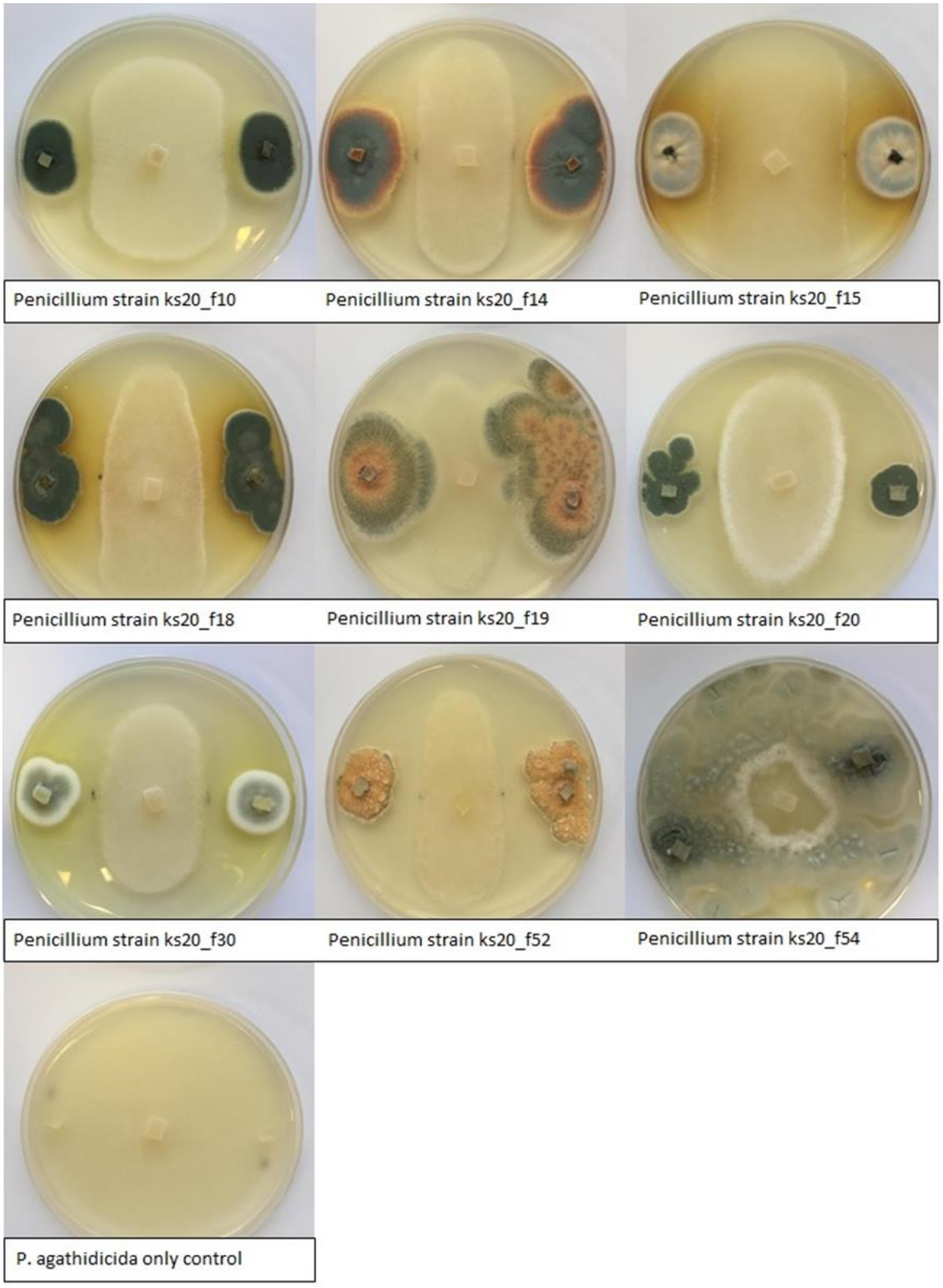

Figure 6.1. The fungal strains that were found to significantly reduce *P. agathidicida* mycelial growth compared to *P. agathidicida* only controls in dual culture bioassays.

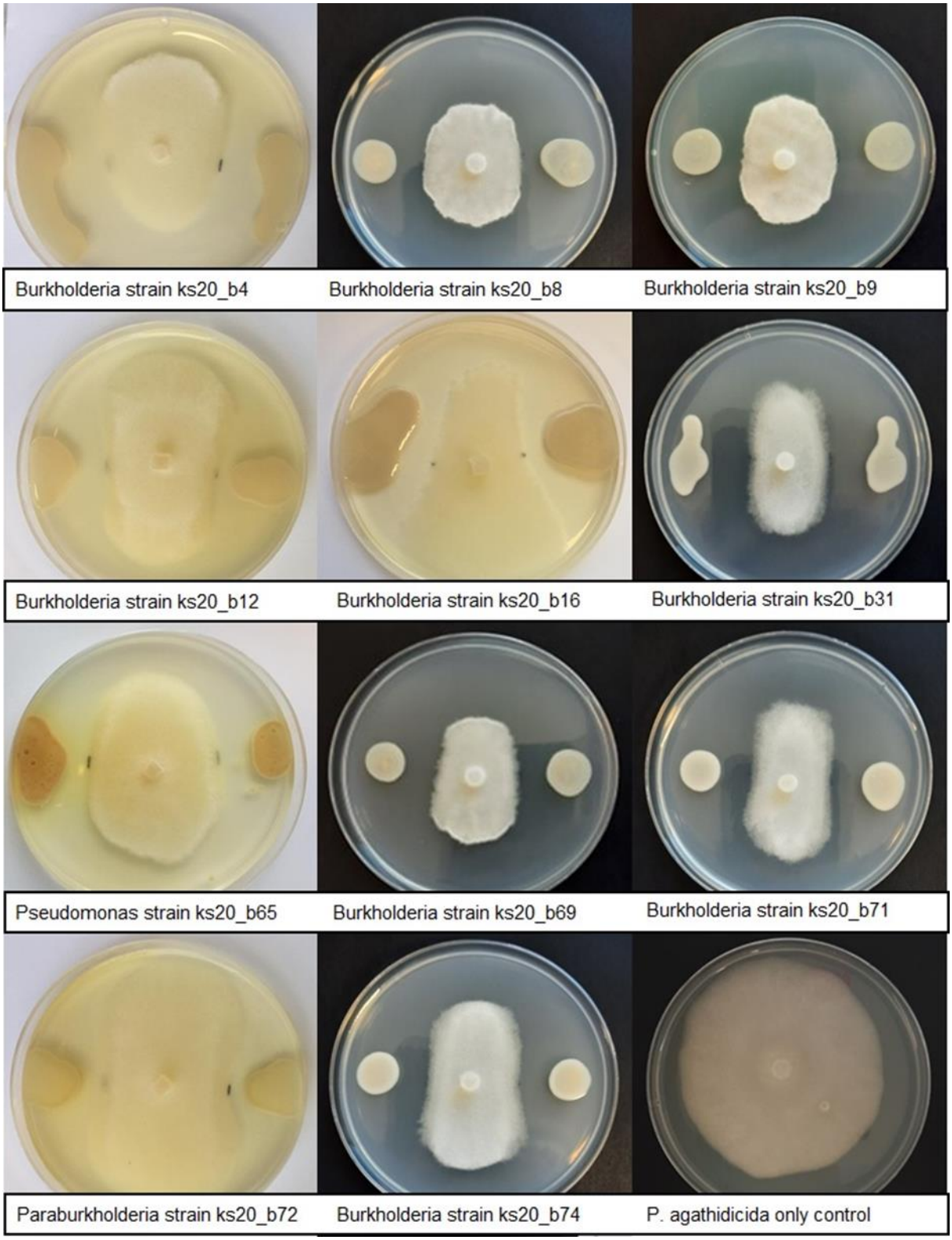

Figure 6.2. The bacterial strains that were found to significantly reduce *P. agathidicida* mycelial growth compared to *P. agathidicida* only controls in dual culture bioassays.

Culture filtrate bioassays

Only one fungal strain, *Penicillium* strain ks20_f20, significantly reduced the mycelial growth of *P. agathidicida* when compared to controls (p-value < 0.001). The mean MI value (%) of *Penicillium* strain ks20_f20 was 33.72 ± 4.95, however *P. agathidicida* cultures treated with this strain's culture filtrate also had a noticeably finer mycelial density (Figure 6.3). Other growth measurements, such as changes to mycelial mass, may be more suited to measure the impact of this strain on *P. agathidicida* growth.

Burkholderia strain ks20_b72 was the only bacterial strain that did not significantly reduce *P. agathidicida* mycelial growth (p-value > 0.05). As shown in Figure 6.5, all other 10 bacterial strains significantly reduced *P. agathidicida* mycelial growth compared to controls (p-value < 0.05). Bacterial strains with the highest mean MI values (%) were *Burkholderia* strain ks20_b9 (16.43 ± 1.71), *Burkholderia* strain ks20_b8 (14.40 ± 2.50) and *Burkholderia* strain ks20_b69 (13.69 ± 3.19). The MI values (mean ± SE) of the culture filtrate bioassays and the results of the Student's T tests are shown in Tables D.1 (fungal strains) and D.2 (bacterial strains), Appendix D.

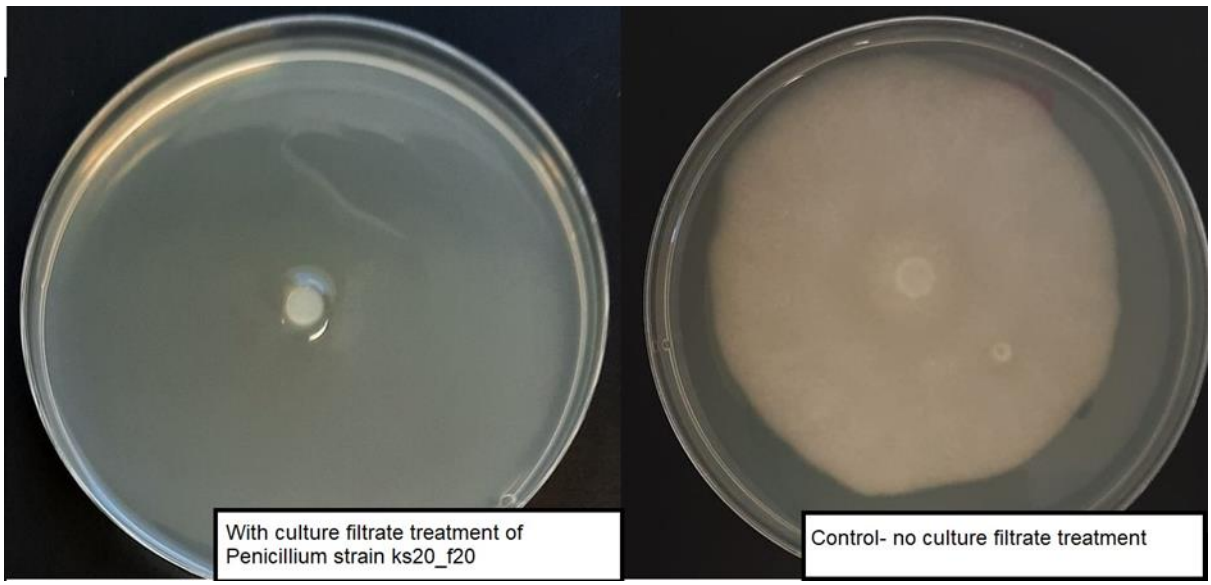

Figure 6.3. The mycelial growth of *P. agathidicida* on day 7 following culture filtrate treatment with *Penicillium* strain ks20_f20 (left) *vs* the mycelial growth of *P. agathidicida* on day 7 that had no culture filtrate treatment (right).

Split plate bioassays

Seven of the nine fungal strains tested significantly reduced *P. agathidicida* mycelial growth compared to controls (p-value < 0.05) (Figure 6.4). Fungal strains with the highest mean MI values (%) were *Penicillium* strain ks20_f15 (20.26 ± 2.64) and *Penicillium* strain ks20_f52 (20.33 ± 4.76). *Penicillium* strain ks20_f20 and *Penicillium* strain ks20_f54 did not significantly reduce *P. agathidicida* mycelial growth compared to controls (p-value > 0.05).

Except for *Pseudomonas* strain ks20_b65, all bacterial strains significantly reduced *P. agathidicida* mycelial growth compared to controls when tested using split plate bioassays (p-value < 0.05, Figure 6.5). The highest mean MI values (%) were found by *Burkholderia* strain ks20_b9 (21.03 ± 3.17) and *Burkholderia* strain ks20_b4 (19.87 ± 4.49). The MI values (mean ± SE) of the split plate bioassays and the results of the Student's T tests are shown in Tables D.1 (fungal strains) and D.2 (bacterial strains), Appendix D.

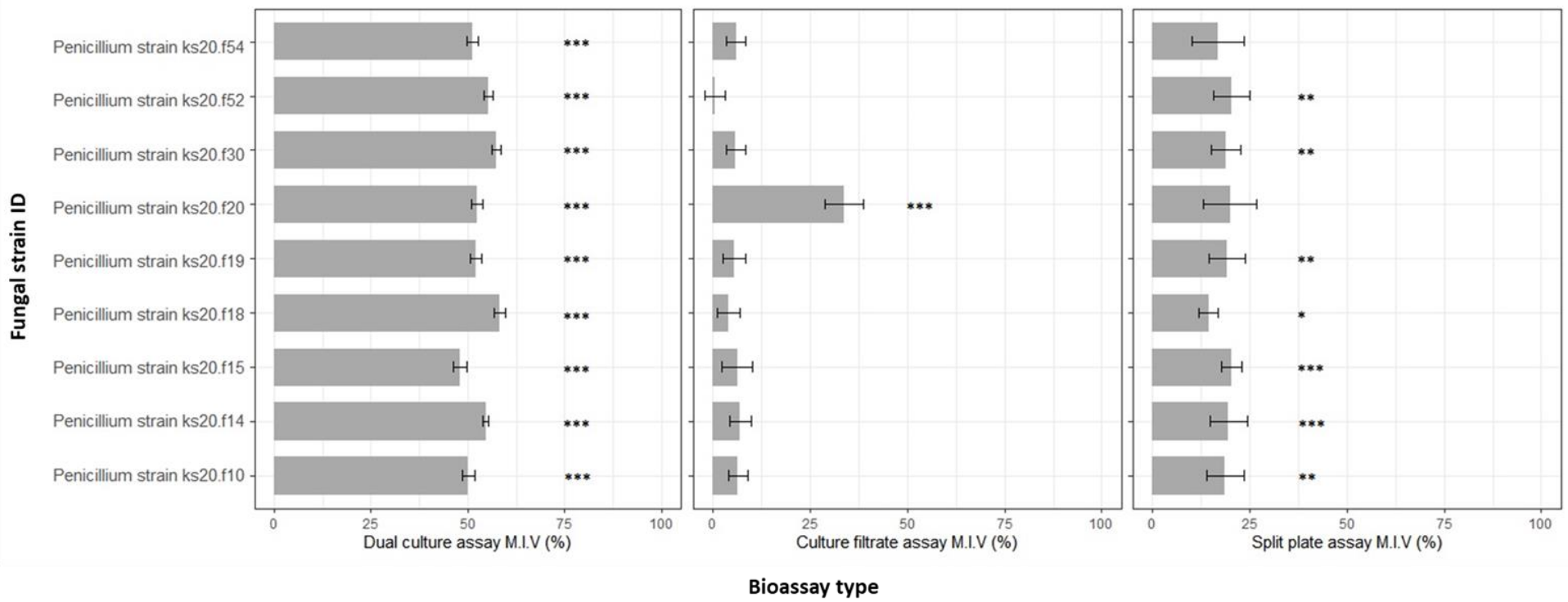

Figure 6.4. The mean ± SE mycelial inhibition (MI) values (%) of the *in vitro* bioassays used to assess the inhibition of fungal strains against *P. agathidicida*. Student T tests were used to identify strains with significantly higher MI values compared to controls (* is p-value < 0.05, ** is p-value < 0.01 and * is p-value < 0.001).**

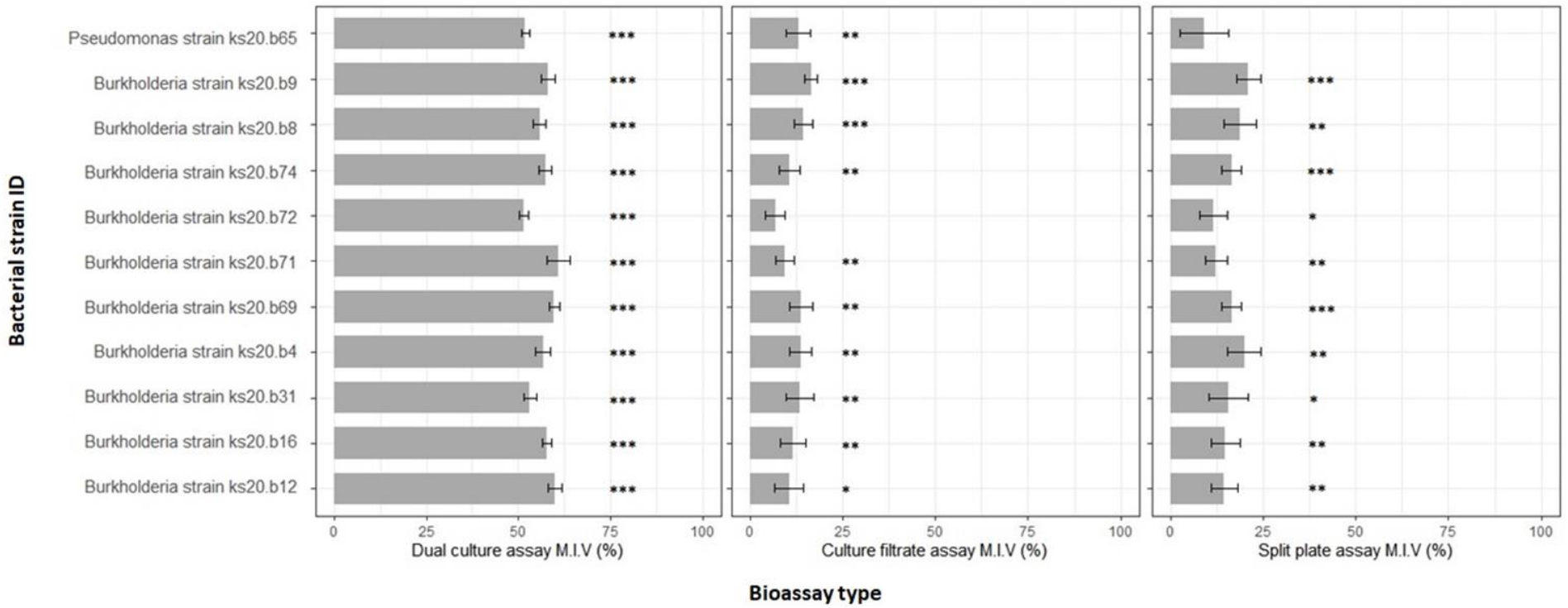

Figure 6.5. The mean ± SE mycelial inhibition (MI) values (%) of the *in vitro* bioassays used to assess the inhibition of bacterial strains against *P. agathidicida*. Student T tests were used to identify strains with significantly higher MI values compared to controls (* is p-value < 0.05, ** is p-value < 0.01 and * is p-value < 0.001).**

6.4.3 VOC profiles of microbial strains

The results of the HS-SPME/GCMS analysis found that *P. agathidicida* produced five VOCs which were identified as 2-phenylethanol; 4-ethylphenol; Methyl salicylate; 2, 2, 4-Trimethyl-1, 3-pentanediol diisobutyrate; and 2, 5-ditert-Butyl-1, 4-benzoquinone. None of these VOCs were produced by any of the fungal or bacterial strains subsequently analysed. Therefore, it is reasonable to suggest that no VOCs produced by the fungal and bacterial strains were also produced by *P. agathidicida* (Figure 6.6 and 6.7).

Across all the fungal strains analysed, 32 different VOCs were produced (Figure 6.6) which covered six chemical classes- sesquiterpenoids, monoterpenoids, ketones, hydrocarbons, fatty alcohols and benzenoids (Figure 6.8). Sesquiterpenoids were the most detected compound type and were detected in six out of the nine fungal strains. *Penicillium* strain ks20_f20 did not produce any VOCs, a finding that is consistent with the results of the split plate bioassays which found that this strain did not significantly inhibit *P. agathidicida* mycelial growth (Figure 6.4). Although *Penicillium* strain ks20_f54 did not significantly inhibit *P. agathidicida* mycelial growth when tested using split plate bioassays (Figure 6.4), three VOCs were identified to be produced by this strain (Figure 5.6). Two of these VOCs, 3-Octanone and 1-Octen-3-ol, were also produced by *Penicillium* strain ks20_f30. Therefore, it is likely that these two VOCs are not responsible for the mycelial inhibition observed by *Penicillium* strain ks20_f30.

Across all the bacterial strains analysed, 29 different VOCs were produced (Figure 6.7) which covered 13 chemical classes- alkenes, benzenoids, epoxides, fatty acyls, hydrocarbons, ketones, monoterpenoids, organic disulphides, organic oxides, organic trisulfides, pyrazines, sesquiterpenoids and thiocarboxylic acids (Figure 6.8). The alkene compound 1-Undecene was detected to be produced by all of the *Burkholderia* strains. Additionally, the pyrazine compound 2-butan-2-yl-3-methoxy-5-(2-methylpropyl) pyrazine had a high relative peak area in several of the *Burkholderia* strains (Figure 6.7). No compounds were produced by *Paraburkholderia* strain ks20_b72 which did significantly inhibit *P. agathidicida* mycelial growth in split plate bioassays (Figure 6.5). Reasons for the absence of VOCs detected to be produced by this strain are unknown. *Pseudomonas* strain ks20_b65 did not significantly inhibit *P. agathidicida* mycelial growth in split plate bioassays and most VOCs produced by this strain were not produced by the other *Burkholderia* strains. The exceptions to this were beta-Myrcene and Dimethyl trisulphide (Figure 6.7) which are unlikely to be responsible for the mycelial inhibition of *P. agathidicida* found by many the *Burkholderia* strains.

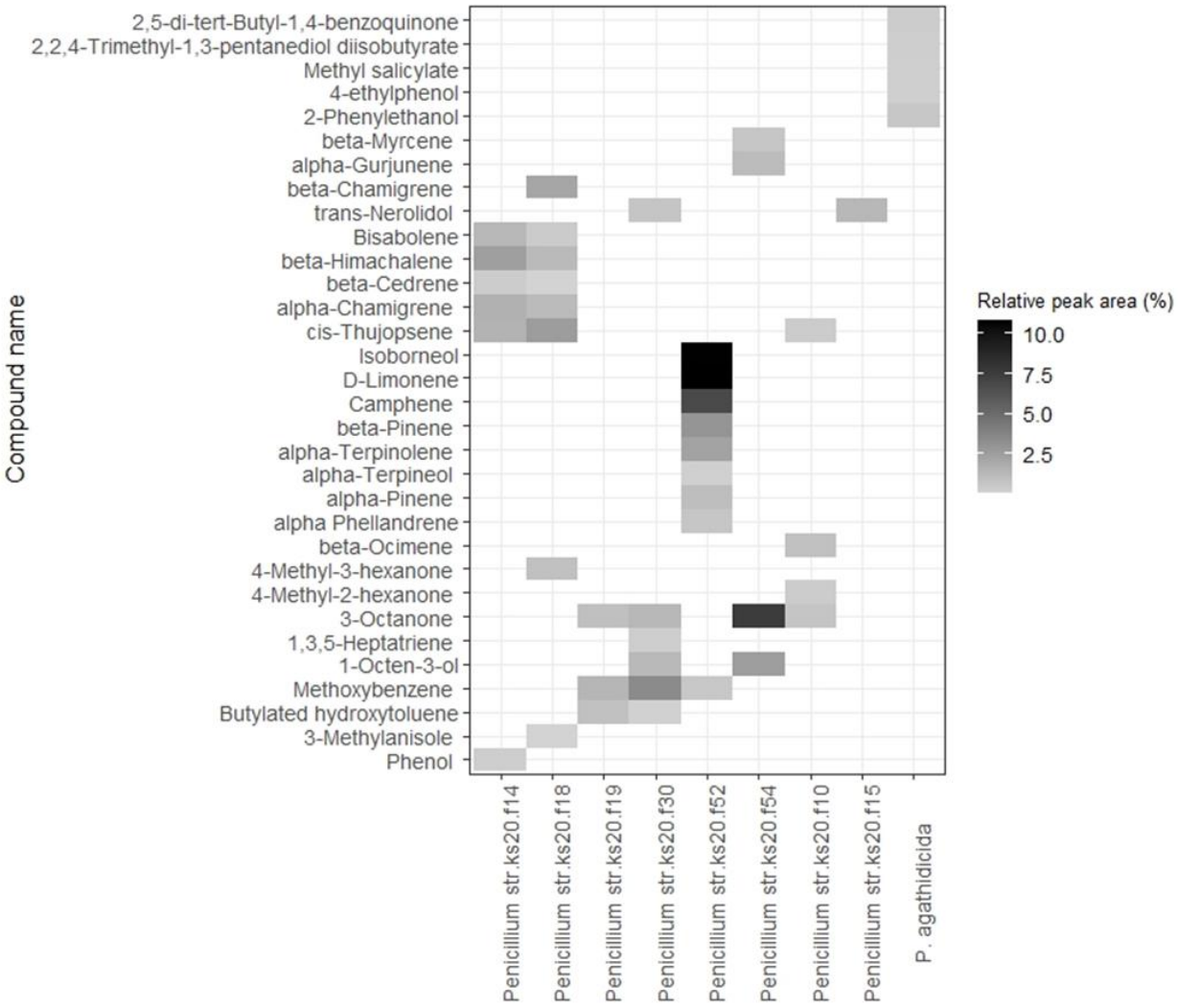

Figure 6.6. The relative peak areas (%) of the VOCs identified to be released by the fungal strains and *P. agathidicida*. *Penicillium* strain ks20_f20 is not shown on this figure as no VOCs were detected to be produced by this strain.

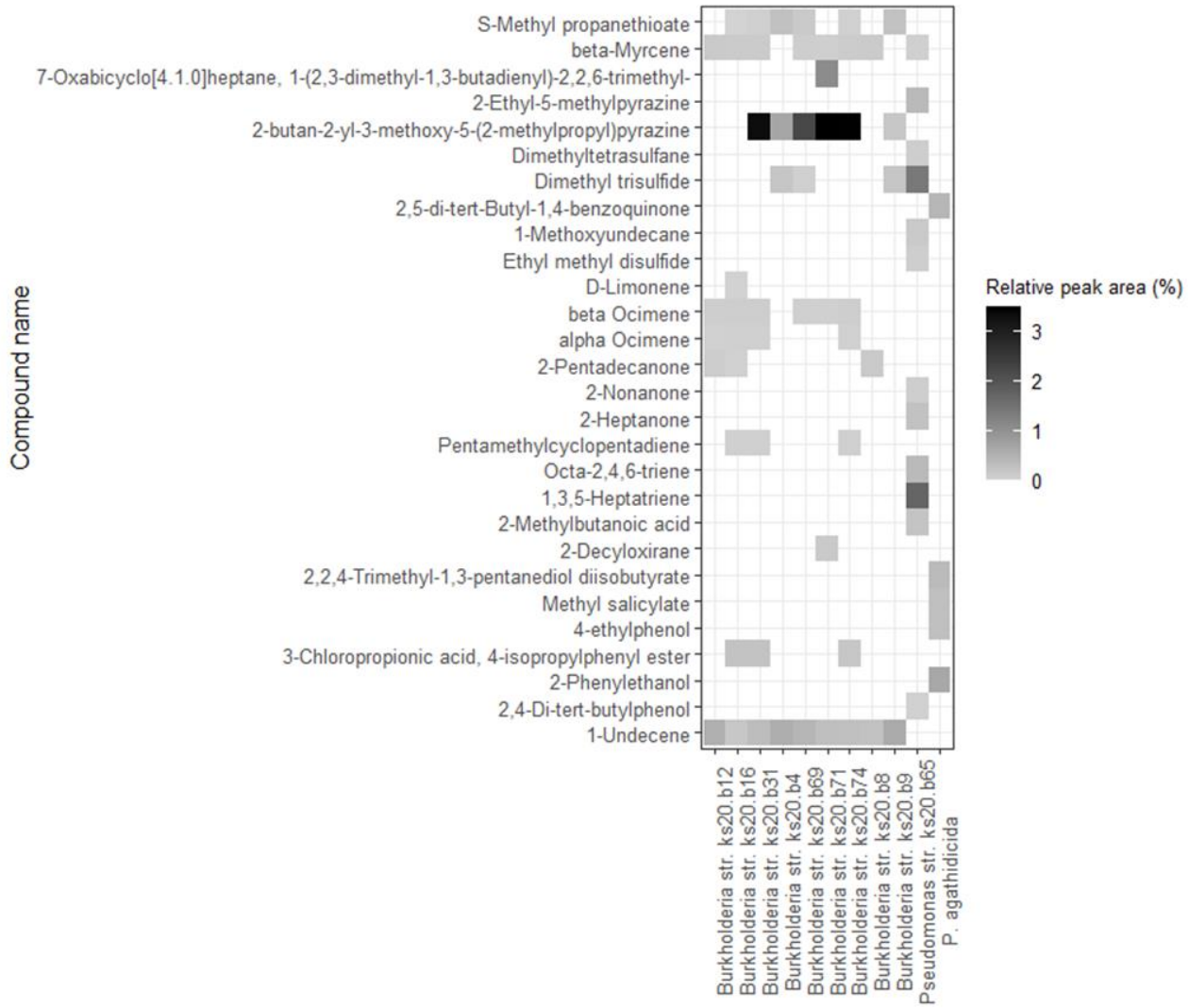

Figure 6.7. The relative peak areas (%) of the VOCs identified to be released by the bacterial strains and *P. agathidicida*. *Paraburkholderia* strain ks20_b72 is not shown on this figure as no VOCs were detected to be produced by this strain.

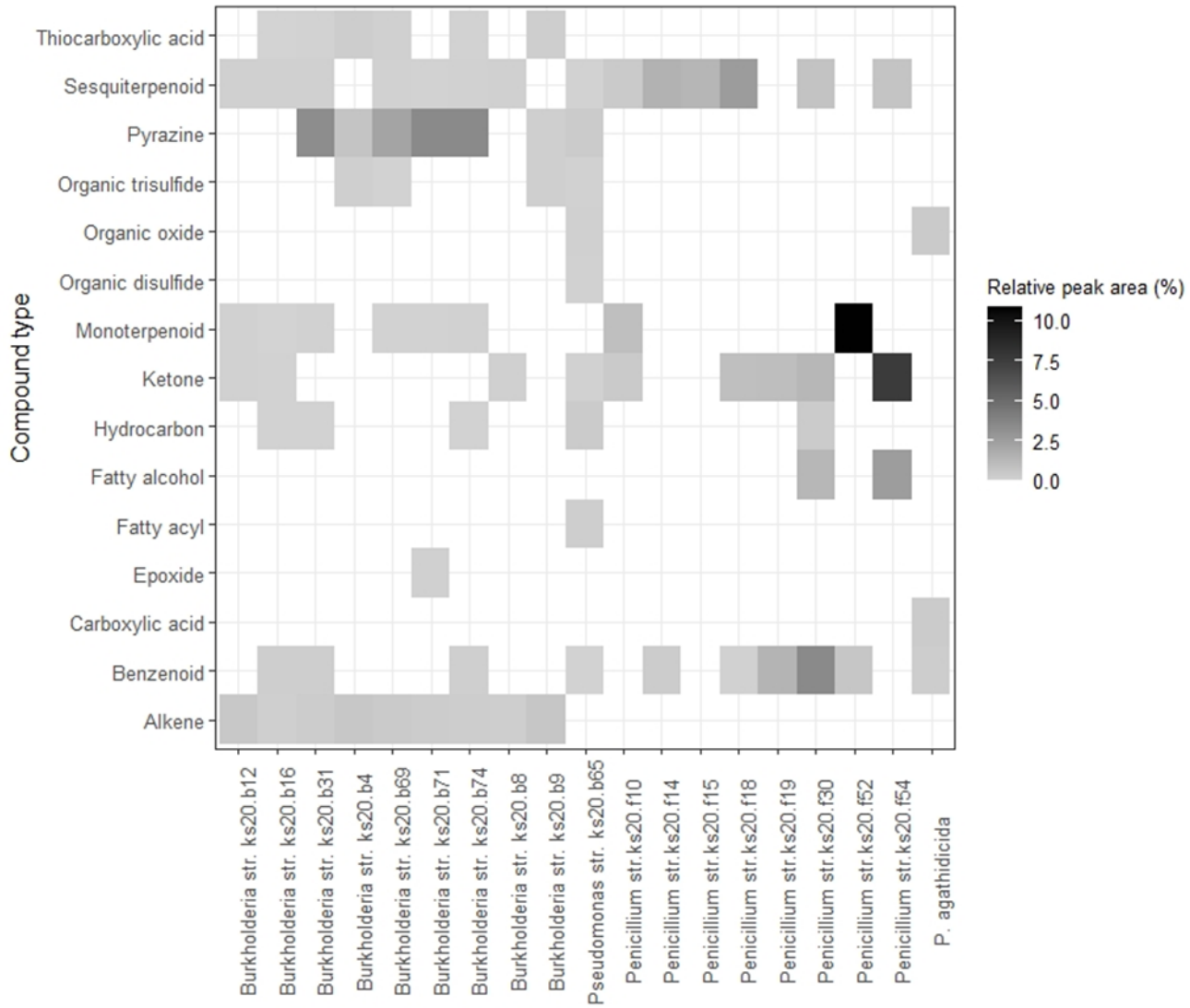

Figure 6.8. The relative peak areas (%) of the chemical classes found to be released by the fungal and bacterial strains based on the profiling and identification of their VOCs.

6.5 Discussion

This study aimed to identify members of the kauri soil microbiota that inhibited the mycelial growth of *P. agathidicida* which could be selected as candidate biocontrol agents for the management of kauri dieback. When tested using dual culture bioassays, microbial strains belonging to the genera *Penicillium*, *Burkholderia, Paraburkholderia* and *Pseudomonas* significantly inhibited the mycelial growth of *P. agathidicida.* Several of these bacterial and fungal strains were also found to inhibit *P. agathidicida* in culture filtrate and split plate bioassays. Despite the inhibition of *P. agathidicida* being lower in culture filtrate and split plate bioassays, findings suggest that the production of diffusible and volatile compounds by several of these strains are factors contributing to inhibition.

6.5.1 *Burkholderia* strains display strong inhibition towards *P. agathidicida*

In each of the three bioassays used to test for inhibition, all nine *Burkholderia* strains significantly inhibited the mycelial growth of *P. agathidicida*. *Burkholderia* species have previously been reported to antagonise a wide range of pathogens, including *Phytophthora* species (Elshafie et al., 2012; Kong et al., 2020; Sopheareth et al., 2013). In the dual culture bioassays, there was a clear zone of inhibition present on agar and no strains were observed to have come into direct contract with *P. agathidicida*. Therefore, it is unlikely that inhibition was a result of direct mycoparasitism or microbial competition on agar. All *Burkholderia* strains significantly inhibited the growth of *P. agathidicida* in split plate and culture filtrate bioassays, albeit at a lower level of inhibition than in dual culture. A similar finding was observed by Elshafie et al. (2012) which found a greater inhibition of *Phytophthora cactorum* by *Burkholderia gladioli* pv. *agaricicola* in dual culture bioassays compared to culture filtrate treatments. Despite inhibition being lower in the culture filtrate bioassays, these results support that diffusible compounds released by the bacterial strains are likely to be contributing to inhibition. This notion is also supported by the clear zones of inhibition observed in the dual culture bioassays, which suggest that inhibitory compounds were being released by the bacteria and diffusing through the agar. The production of secondary metabolites such as siderophores, antibiotics, hydrolytic enzymes and biosurfactants are known to contribute to the suppression of pathogenic fungi by bacteria (Neeraja et al., 2010). Furthermore, species belonging to *Burkholderia* have been demonstrated to produce a wide range of anti-microbial secondary metabolites (Depoorter et al., 2016; Li et al., 2002; Vial et al., 2007). Further research that characterises the secondary metabolites produced by the *Burkholderia* strains, both on solid agar and in culture filtrate form, is required to understand the mechanisms driving inhibition of *P. agathidicida*. For example, Caulier et al. (2018) used different forms of *in vitro* assays to assess the

production of enzymes, biosurfactants and siderophores on solid media by bacterial strains showing antagonism towards *P. infestans*.

The only *Pseudomonas* strain isolated in this study (*Pseudomonas* strain ks20_b65) did not significantly inhibit *P. agathidicida* when tested using split plate bioassays. Despite being found to produce a diverse range of VOCs by the HS-SPME/GCMS analysis, *Pseudomonas* strain ks20_b65 is unlikely to be producing any that inhibit the growth of *P. agathidicida* based off the results of the split plate bioassays. Similarly, the culture filtrates of *Paraburkholderia* strain ks20_b72 did not significantly inhibit *P. agathidicida* therefore it is unlikely to be producing any inhibitory diffusible compounds. Although these two strains showed strong inhibition of *P. agathidicida* in dual culture, they may be of lesser interest for further research compared to the strains of *Burkholderia* isolated in this study.

6.5.2 *Penicillium* strains were variable in their inhibition of *P. agathidicida*

This study identified several strains of *Penicillium* that significantly inhibited *P. agathidicida* in dual culture bioassays. The results of this study align with the findings of Chapter 4, which identified that the OTU abundance of *Penicillium* was significantly higher in asymptomatic kauri soils. Members of the *Penicillium* genus are widespread in soils and have been demonstrated to antagonise a variety of plant pathogens (Nicoletti & De Stefano, 2012). In addition, *Penicillium* spp. are well adapted and highly competitive members of the soil environment (Nicoletti & De Stefano, 2012). Therefore, the identification of *Penicillium* strains showing inhibition towards *P. agathidicida* is promising in regards to their practical application in the field. Quite often, microbial antagonists fail to confer disease suppression when applied in the field as they cannot compete with the resident soil microbiota to successfully establish within the rhizosphere (Expósito et al., 2017). When screened in dual culture, several of the *Penicillium* strains overgrew the mycelium of *P. agathidicida* (Figure D.3, Appendix D). This suggests that many of the *Penicillium* strains are inhibiting *P. agathidicida* by having a more competitive growth rate and resource use on agar which is physically limiting the growth of *P. agathidicida* (Bunbury-Blanchette & Walker, 2019). For example, *Penicillium* strain ks20_f54 was only found to significantly inhibit *P. agathidicida* in dual culture bioassays, thus it is unlikely to be producing anti-microbial VOCs or diffusible compounds that can inhibit *P. agathidicida*.

Culture filtrate treatment of *Penicillium* strain ks20_f20 strongly inhibited *P. agathidicida*. Furthermore, this strain produced a clear zone of inhibition in dual culture which suggests that it may be producing diffusible compounds that can inhibit *P. agathidicida*. As with strains of *Burkholderia*, further research is required to characterise the diffusible compounds produced by

Penicillium strain ks20_f20. For example, Ma et al. (2008) and Ma et al. (2013) used thin layer chromatography (TLC) to isolate compounds from the liquid cultures of *Penicillium striatisporum* that were identified to inhibit the mycelial growth and zoospore germination of *P. capsici*.

6.5.3 Contribution of microbial VOCs to the inhibition of *P. agathidicida*

All the *Burkholderia* strains inhibited the growth of *P. agathidicida* in split plate bioassays, suggesting they may be releasing VOCs with inhibitory properties against *P. agathidicida.* The compound 1-Undecene was produced by all of the *Burkholderia* strains. Hunziker et al. (2015) identified 1-Undecene to inhibit the mycelial growth, sporangia formation and zoospore release of *Phytophthora infestans* when produced by antagonistic strains of *Pseudomonas*. Another VOC produced by several of the *Burkholderia* strains was 2-butan-2-yl-3-methoxy-5-(2-methylpropyl) pyrazine. Although this VOC has not been studied for its anti-microbial properties, other pyrazine VOCs (i.e. 2, 5-dimethyl pyrazine and 2-methoxy-3-methyl pyrazine) have previously been identified to inhibit the growth of *P. infestans* and *P. capsici* (Lazazzara et al., 2017; Munjal et al., 2016). In addition, S-Methyl propanethioate was produced by many of the *Burkholderia* strains in this study. However, based on the literature research of this study, the anti-microbial properties of this compound against plant pathogens such as *Phytophthora* have not yet been reported. Several of the *Burkholderia* strains produced the monoterpenoid compounds alpha Ocimene and beta Ocimene. Ocimene compounds were found to be produced by strains of *Burkholderia tropica* that showed inhibition against several plant pathogens, although inhibition was not tested for *Phytophthora* (Tenorio-Salgado et al., 2013). In addition, Ocimene compounds were identified as components of plant essential oils that were able to significantly inhibit the mycelial growth of *P. capsici, P. drechsleri* and *P. melonis* (Amini et al., 2016).

Most of the *Penicillium* strains isolated in this study significantly inhibited *P. agathidicida* in split plate bioassays. Many of the VOCs produced by these strains were identified as terpenoids, ketones and benzenoids. In particular, *Penicillium* strain ks20_f14 and *Penicillium* strain ks20_f18 produced a range of sesquiterpenoids and *Penicillium* strain ks20_f52 produce a range of monoterpenoids. However, the scientific knowledge regarding the inhibitory roles of VOCs produced by *Penicillium* against fungal or oomycete plant pathogens is limited. *Penicillium* species are better researched for their production of anti-bacterial compounds rather than anti-fungal compounds (Rouissi et al., 2013). In addition, unlike the strains of *Burkholderia*, no VOCs were found to be consistently produced by the *Penicillium* strains that inhibited *P. agathidicida*.

6.6 Conclusion

This study has identified strains belonging to the genera *Burkholderia* and *Penicillium* that warrant further research as candidates for the biocontrol of *P. agathidicida.* As all strains of *Burkholderia* were able to inhibit *P. agathidicida* mycelial growth without establishing direct contact, the mode of inhibition is likely to be through production of diffusible and volatile inhibitory compounds. Many of the *Penicillium* strains had a fast growth rate which restricted *P. agathidicida* growth on agar, suggesting that microbial competition is a likely mode of inhibition. HS-SPME/GCMS analysis identified that several VOCs, such as 1-Undecene, were produced by many of the *Burkholderia* strains. Further research is required to measure the impact of these VOCs in their pure form on the growth of *P. agathidicida.* In addition, cultures filtrates of all *Burkholderia* strains and one *Penicillium* strain inhibited the growth of *P. agathidicida.* The diffusible compounds present in these culture filtrates need to be identified so that the direct impact of these compounds on the growth of *P. agathidicida* mycelia can be assessed.

6.7 Acknowledgements

This project was supported by the New Zealand Tertiary Education Commission CoRE grant to the Bio-Protection Research Centre. Thanks to Jason Breitmeyer, Lincoln University, for providing technical support in performing and processing the HS-SPME/GCMS analysis as well as advice on how to quality control and manage the data outputs. Thanks to Te Roroa Iwi for permitting access to Waipoua Forest, for without this these microbial strains would never have been discovered.

Chapter 7

General discussion

7.1 Introduction

My PhD research aimed to characterise the taxonomic diversity of the kauri soil microbiota, whilst studying their response to dieback disease and identifying if any members exhibit antagonism to *Phytophthora agathidicida*. The findings of my research have identified candidate microbial antagonists against *P. agathidicida*, which warrant further research for their potential use in the management of kauri dieback. By studying how kauri dieback impacted upon properties of the soil microbiota, my research highlighted the secondary impacts of dieback which extend beyond initial tree disease expression and mortality. Additionally, my research has begun to examine the potential impacts of pine plantation establishment on the soil microbiota across Waipoua Forest (Northland Region, New Zealand). These findings will facilitate future research that assesses the influence of historical forestry disturbances on the spread of kauri dieback across fragmented kauri forests in New Zealand. This final chapter outlines the main findings and implications of my PhD research. In addition, the limitations of the scope of my research are discussed and recommendations for future work are proposed.

7.2 Summary of Findings

7.2.1 Pine plantation establishment has significantly altered the soil microbiota surrounding remnant kauri forests (Chapter 3)

Insights:

The findings of **Chapter 3** identified significant differences in the diversity and composition of microbial communities between kauri and pine forest soils, which significantly correlated to differences in soil chemical properties such as bioavailable N, organic matter and total carbon (C). Pine soils were found to have a significantly lower relative abundance of microbial taxa that have previously been reported to function in supporting plant health such as *Rhizobiaceae*, *Trichoderma* and *Pseudomonas* (Avis et al., 2008; Gaiero et al., 2013; Kumar et al., 2017). However, the ecological and functional roles of these taxa within kauri forest soils has yet to be defined and requires further investigations before we can draw any conclusions. **Chapter 3** has provided evidence to suggest that pine plantation establishment has altered the soil microbiota surrounding remnant kauri forests. These findings will be an important contribution to studies that assess the impact of historical forestry disturbances on the health of kauri forests and their susceptibility to kauri dieback.

Limitations and recommendations for future research:

Chapter 3 performed high throughput sequencing on microbial DNA to identify differences in the composition and relative abundances of microbial taxa in kauri and pine forest soils. Although effective for the primary purposes of the study, such methods did not provide quantitative measurements on the absolute abundances of the metabolically active members of the soil microbiota (Bang-Andreasen et al., 2020; Cox et al., 2017). This limitation is also shared with the studies presented in **Chapter 4** and **5**, which similarly performed high throughput sequencing on microbial DNA. To address this issue, future studies could instead perform high throughput sequencing on microbial RNA to more accurately quantify differences in the taxonomic abundances of the metabolically active soil microbial communities (Cox et al., 2017; Tviet et al., 2014; Young et al., 2018).

Chapter 3 identified differences in the taxonomic diversity of the soil microbiota between kauri and pine soils. However, more work is required to identify differences in the functional diversity of the soil microbiota associated with kauri and pine soils. Understanding the impacts of pine plantation establishment on soil microbial function is important because the functional diversity of the soil microbiota can reflect their ability to respond to soil disturbances (Pignataro et al., 2012). Proposed methods may include measurements of soil respiration and soil enzyme activities (Nkongolo & Narendrula-Kotha, 2020), functional gene arrays (Zhou et al., 2010) and quantification of targeted functional genes using qPCR (Trivedi et al., 2019).

Furthermore, **Chapter 3** compared differences in the soil microbiota between kauri and pine forest stands. However, there are a variety of land use types surrounding primary kauri fragments in Waipoua Forest such as pasture, secondary and regenerating kauri forest, and kauri nurseries. More comprehensive assessments on the impacts of anthropogenic disturbances on the soil microbiota across Waipoua Forest will require characterisation of the resident soil microbiota associated with these different forms of land use.

7.2.2 Significant differences were found in the soil microbial communities associated with asymptomatic and symptomatic kauri (Chapter 4)

Insights:

Chapter 4 identified significant differences in the composition of fungal and bacterial communities between asymptomatic and symptomatic kauri soils. The fungal diversity of symptomatic kauri soils was significantly higher than asymptomatic kauri soils, which may be a response to the increased outputs of necrotic plant litter attracting an influx of secondary colonising fungi. Several microbial taxa were found in significantly higher abundance in asymptomatic kauri soils which have previously

been reported as microbial antagonists against *Phytophthora* pathogens, such as *Penicillium*, *Trichoderma* and *Pseudomonas* (Bae et al., 2016; Caulier et al., 2018; Ma et al., 2008). Although requiring more research, the association of these taxa with asymptomatic kauri could suggest their potential as examples of disease suppressive microbial agents of kauri dieback.

Long term, the cascading impacts of widespread forest dieback can cause forests to experience increased C losses and reduced C uptake (Avila et al., 2016). **Chapter 4** identified significant differences in the composition of microbial genes related to C cycling between asymptomatic and symptomatic kauri soils. Moreover, symptomatic soils had a greater number of C degradation genes found in a higher relative abundance. These shifts in C degradation genes highlight the potential secondary impacts of kauri dieback on the functional potential of the soil microbiota. Although non-significant, values for total C were higher in symptomatic kauri soils. Over time the increased abundance of carbon degradation genes in symptomatic soils may result in increased soil C losses. Kauri forest soils are incredibly C dense and kauri contribute to most of these C inputs (Macinnis-Ng & Schwendenmann, 2015). Therefore, increased rates of microbial C degradation following outbreaks of kauri dieback would greatly impact long term nutrient cycling dynamics in kauri forests. Future studies should repeat these assessments of soil microbial function in dieback afflicted kauri forests over longer time frames to improve our understanding on the long term impacts of kauri dieback.

Limitations and recommendations for future research:

Chapter 4 analysed soils collected from a single kauri forest in New Zealand (Northland Region, New Zealand). Although replication was performed across four different sites within Waipoua Forest, findings should be validated by characterising the soil microbiota of asymptomatic and symptomatic kauri sampled from several different kauri forests across New Zealand. For example, dieback is reported to have four major disease foci in New Zealand: - Waipoua Forest (Northland), the Waitakere Ranges (Auckland), Punaruku (Northland), and Great Barrier Island (Hauraki Gulf, Auckland) (Beauchamp, 2013; Waipara et al., 2013). Future studies could sample kauri soils from each of these four forest regions to identify if the differences found in the soil microbiota between asymptomatic and symptomatic kauri is consistent across a larger geographical scale.

Additionally, no repeated sampling was performed at different time points to validate the findings of the first sampling round. Further sampling from the same asymptomatic and symptomatic kauri is needed with samples analysed using the same methods presented in **Chapter 4**. The spread and expression of *Phytophthora* diseases can be influenced by environmental differences across seasons, such as temperature and rainfall (Burgess et al., 2019; Sánchez et al., 2002). Furthermore, the

composition of fungal (Voříšková et al., 2014) and bacterial (López-Mondéjar et al., 2015) soil communities can be influenced by seasonality. Therefore, sampling should be repeated during the same season as the first sampling round (autumn) to avoid differences in seasonality limiting comparison between repeated sampling rounds. However, once the findings of the **Chapter 4** have been validated, future studies may wish to perform soil sampling across different seasons to investigate how differences in environmental conditions impact the response of the soil microbiota to kauri dieback disease.

Several other limitations of the study described in **Chapter 4** have been previously described in more detail in **Section 5.2**. In brief, it was not possible to sample soils from symptomatic kauri that were infected with consistent inoculum loads of *P. agathidicida* and the time since initial infection of symptomatic kauri could not be accurately determined. **Chapter 5** aimed to address these limitations by using a seedling infection study to examine the response of the soil microbiota to dieback disease expression under more controlled environmental conditions. The findings and limitations of this study are discussed in the next section.

7.2.3 Strong shifts in soil bacterial communities following infection of kauri seedlings with *P. agathidicida* (Chapter 5)

Insights:

Chapter 5 identified that soils from inoculated kauri seedlings had a significantly higher bacterial diversity and biomass, as well as a significantly different bacterial community composition compared to non-inoculated seedlings. In contrast, soil fungal communities showed no significant differences in their diversity or composition between inoculated and non-inoculated seedlings. Inoculated kauri seedlings had a significantly lower fungal: bacterial biomass which may be due to an increased abundance of more competitive and well-adapted bacterial taxa following seedling infection. Following plant death, soil bacteria are often the initial colonisers due to their ability to utilise readily available sugars (Greaves, 1971) and it is not until the later stages of plant decay that populations of soil fungi rise (Prewitt et al., 2014).

The findings of **Chapter 5** identified several bacterial taxa that were found in significantly higher relative abundance in soils from inoculated seedlings which included several members of the Firmicutes, such as *Bacillus* and *Paenibacillus*. Following pathogen attack, plants can stimulate the enrichment of beneficial microbiota which function to support disease suppression and promote plant growth (Bakker et al., 2018; Berendsen et al., 2018). Thus, the increased abundance of the aforementioned bacterial taxa in soils from inoculated seedlings may suggest they are forming part of a protective response to seedling infection. Berendsen et al. (2018) identified bacterial taxa that

increased in abundance following downy mildew infection of *Arabidopsis thaliana* and demonstrated that co-inoculations of these taxa were able to induce plant systemic resistance and suppress disease upon further infection. For further studies aiming to validate the findings of **Chapter 5**, the bacterial taxa that showed strong responses to seedling infection could be amended to a higher soil population density. Kauri seedlings grown in these amended soils could be then be infected with *P. agathidicida* to identify if the increased abundance of these bacterial taxa suppressed dieback disease expression.

Limitations and recommendations for future research:

Although **Chapter 5** aimed to build on the findings of **Chapter 4**, the major findings of the two studies did not correspond. **Chapter 5** found pronounced differences in soil bacterial communities and only minor differences in soil fungal communities between inoculated and non-inoculated seedlings, whereas **Chapter 4** identified pronounced differences in soil fungal communities between asymptomatic and symptomatic kauri. The proposed reasons for these differences between the two studies were outlined in detail in **Section 5.5.5**. To summarise, the experiment performed in **Chapter 5** selected 18-month-old kauri seedlings as a plant host for infection with *P. agathidicida*. The identity and functional traits of dominant trees are known to impact the abundance and composition of soil fungal communities (Urbanová et al., 2015). Due to their smaller root systems and plant litter outputs, the kauri seedlings are likely to have a smaller influence on soil microbiota when compared to the larger, mature kauri (approximately 200 years old) studied in **Chapter 4**. Thus, seedling disease expression may not have elicited the same response of the soil fungal communities as would have occurred when they were associated with a mature kauri host. Furthermore, the low plant litter outputs of kauri seedlings and the physical restrictions imposed by using a closed container pot trial may have restricted large population fluxes of secondary colonising fungi following seedling disease expression. Based on these limitations, future studies should examine the response of the soil microbiota to kauri seedling infection using controlled field trials on more mature kauri trees, rather than using smaller scale seedling pot experiments. This would examine the response of the soil microbiota to kauri infection and standardise variables, such as the inoculum load of *P. agathidicida* and time of infection, whilst being more compatible the field studies conducted in **Chapter 4**.

In **Chapter 5**, a six-week inoculation period was selected before terminating the seedling experiment as the aim was to identify microbial groups that were the primary respondents to seedling infection. However, this may have contributed to the more pronounced response of soil bacterial communities to seedling infection in comparison to soil fungal communities. As already discussed, soil bacteria are

often the initial colonisers of dead plant tissue and soil fungal populations do not rise until the later stages of decay (Prewitt et al., 2014). For us to better understand changes in microbial community dynamics following kauri infection, future studies should select a longer time period (+6 months) over which to study the response of the soil microbiota to seedling infection. In addition, a single time point was selected for soil sampling as this was sufficient to detect the broad taxonomic differences in the microbial communities of inoculated and non-inoculated seedlings. However, future studies may wish to perform repeated sampling over the course of the infection period (i.e. 6, 12, 18, 24 weeks). This would allow for a time series analysis to be performed so we could better understand the successional changes in microbial community dynamics following seedling infection.

7.2.4 *In vitro* bioassays identify *Burkholderia* and *Penicillium* strains as promising antagonists of *P. agathidicida* (Chapter 6)

Insights:

The findings of **Chapter 6** identified 11 bacterial strains, the majority of which were identified as *Burkholderia* sp., and nine fungal strains, which were all *Penicillium* sp., that significantly inhibited *P. agathidicida* mycelial growth. The results of culture filtrate and split plate bioassays identified that several of these strains, particularly the *Burkholderia* strains, appear to be inhibiting *P. agathidicida* through the production of diffusible and volatile organic compounds. Although further research is required to fully define their mechanisms of inhibition, this research has identified several strains which show promise as potential antagonists of *P. agathidicida*.

Limitations and recommendations for future research:

Chapter 6 performed non-targeted isolations of microbial strains from soil to obtain strains for screening with *P. agathidicida*. In **Chapter 4,** several microbial taxa were found in significantly higher relative abundance in asymptomatic kauri soils (i.e. *Penicillium*, *Trichoderma*, *Pseudomonas,* and Enterobacteriaceae). Promisingly, **Chapter 6** did identify several of these taxa (*Penicillium* and *Pseudomonas*) to inhibit *P. agathidicida* which supports the findings of **Chapter 4**. To more fully assess if the microbial taxa found in significantly higher abundance in asymptomatic kauri soils exhibit disease suppressive properties, strains of these taxa need to be isolated in a targeted manner and screened against *P. agathidicida*.

The inhibition of *P. agathidicida* by each strain was evaluated by assessing their impact on the mycelial growth of *P. agathidicida*. However, their potential to inhibit *P. agathidicida*'s sporangia production and zoospore release needs to be quantified. Such measurements are required because it is the motile zoospores released by *Phytophthora* pathogens that are responsible for plant infection (Bellgard et al., 2016). The limitations of this choice of measurement were evident when

assessing inhibition of *P. agathidicida* after culture filtrate treatment with *Penicillium* strain ks20_f20. Measurements of mycelial diameter did not reflect the large reductions in *P. agathidicida* mycelial density that were also observed following treatment with this strain. Performing additional measurements (i.e. their impact on mycelial mass) may provide a more accurate assessment of how each strain is impacting the growth of *P. agathidicida*.

It is important to fully understand the mode of action and mechanism of control of each strain before they can be appropriately and effectively applied in the field for biocontrol (Spadaro & Gullino, 2005). Therefore, further research is required to characterise the diffusible compounds released by the strains whose culture filtrate inhibited *P. agathidicida* (Frisvad et al., 2013; Nandhini et al., 2018; Sophеareth et al., 2013).

Effective biocontrol agents should ideally be highly host specific to minimise their impacts on non-target organisms and prevent their efficacy being compromised by alternate hosts (Stiling & Cornelissen, 2005). Therefore, the potential deleterious effects of the microbial strains on kauri and the co-occurring plant species of kauri forests when applied to soils in larger loads needs to be evaluated. Many of the antagonistic traits of biocontrol agents against soil pathogens, such as competition for nutrients and the release of anti-microbial metabolites, can also impact the activity and function of the resident soil microbiota (Cordier & Alabouvette, 2009; Scherwinski et al., 2008). Therefore, further research should aim to identify if the strains identified in **Chapter 5** impact the kauri soil microbiota when applied to soils at higher loads.

The findings of **Chapter 6** identified strains which effectively inhibited *P. agathidicida* growth *in vitro*. However, *in vivo* studies need to be performed to identify if these strains can effectively supress pathogen establishment and the expression of kauri dieback in soils inoculated with *P. agathidicida*. If these strains demonstrate effective biocontrol activity against *P. agathidicida in vivo* and have been identified to have no deleterious impacts on non-target organisms, studies which assess their ability to suppress dieback in the field may wish to proceed. Furthermore, combining different strains of microbial antagonists can often provide a higher level of disease suppression as multiple different modes of antagonism are acting against the target pathogen. Thus, further research may also wish to assess the synergistic effects of the microbial strains identified in **Chapter 6** against *P. agathidicida* (Spadaro & Gullino, 2005).

7.3 Conclusion

My PhD thesis is the first study to have fully characterised the fungal and bacterial diversity of the kauri soil microbiota using high throughput sequencing techniques. The findings of my research have

identified that pine plantation establishment has altered the soil microbiota surrounding remnant kauri forests, which will be important for our understanding of how historical forestry disturbances have impacted the health of kauri forests. By comparing differences in the soil microbiota between asymptomatic and symptomatic kauri, my research has identified microbial taxa which may be associated with potentially disease suppressive soils. Furthermore, by comparing differences in microbial gene function between asymptomatic and symptomatic kauri, my research has identified the potential long-term impacts of dieback on soil C and N cycling in kauri forests. In addition, I have identified several strains belonging to the genera *Burkholderia* and *Penicillium* that inhibited the mycelial growth of *P. agathidicida* when tested *in vitro.* These microbial strains require further study to assess their potential to function as biocontrol agents for the management of kauri dieback.

7.4 The instrinsic value of soil biodiversity for New Zealand's kauri forests

Declared a forest sanctuary in 1952, Waipoua Forest was one of the first natural areas to be legally protected in New Zealand (Steward & Beveridge, 2010; Burns & Leathwick, 1996). Waipoua Forest is the largest remnant tract of unmodified kauri in New Zealand (Burns & Leathwick, 1996), and has a high diversity of other native tree and plant species such as *Leptospermum scoparium* and *Dracophyllum robustum*. Unlike the earlier recognition of their botanical value, the belowground diversity of kauri forests has been ignored. Understanding such biodiversity is important, as the soil microbiota drive soil processes, support the provision of ecosystem services, and function as bio-indicators of soil health in response to environmental change (Fierer et al., 2021). The maintainence of soil health and functioning is vital to allow the continued delivery of soil ecosystem services (Adhikari & Hartemink, 2016). The natural capital provided to humans by soil ecosystem services has attracted much research attention across a range of ecosytems (Samarasinghe & Greenhalgh, 2013; Brady et al., 2015; Cram et al., 2015). Thus, the value of the soil microbiota can often be viewed under the spotlight of their natural capital. However, what is often overlooked is the instrinic, non-humanistic biodiversity value of the soil microbiota (Alho et al., 2008). For New Zealand's kauri forests which have previously been exploited for human benefit, the instrinsic value of their biodiversity is something we must continue to protect alongside the support of their ecosystem services. This PhD research identified that the fungal and bacterial diversity of kauri forests is impacted by both land-use change and disease outbreak. In **Chapter 4**, we outlined potential consequences these impacts may have on regulatory ecosystem services, such as forest carbon sequestration. However, we must also highlight that these disturbances have impacted populations of indigenous soil microorganisms that have evolved in these ancient kauri forests and possess a high instrinsic biodiversity value.

7.5 The value and contribution of mātauranga Māori to kauri dieback research

This PhD research was completed in collaboration with *Te Roroa Iwi*, the owners of Waipoua Forest Land and the *kaitiaki* (guardians) of Waipoua Forest sanctuary. Prior to commencing this PhD research, *Te Roroa* permitted us access to Waipoua Forest, without such permissions this research would never have been able to proceed. The sharing of *mātauranga Maori* by *Te Roroa* guided the discovery of suitable forest sites containing asymptomatic and symptomatic kauri. Given the vast size and complex structure of Waipoua Forest, identifying suitable forest sites and performing soil sampling could potentially have been incredibly complex and time consuming. However, by sharing their traditional ecological knowledge of Waipoua, *Te Roroa* were pivotal in allowing this project to progress, develop, and succeed. I would like to offer an extended gratitude to Tom Donovan and Snow Tane for guiding me directly on site to kauri trees they knew to have tested positive for *P. agathidicida*. The health of kauri forests is linked to the *mauri* (spirit, essence) and *mana* (respect, authority, status, spiritual power) of *Te Roroa*'s communities (Lambert et al., 2018). *Mātauranga Māori* has long recognised the importance of managing kauri dieback using a holistic approach, emphasising how biodiversity, environmental soil conditions, sunlight, and levels of human activity are essential factors controlling the wider health of kauri forests (Shortland et al., 2011; Lambert et al., 2018). The approaches promoted by *mātauranga Māori* directed this PhD research, inspiring us to assess how adjacent land uses and the microbial ecology of kauri soils are important factors for kauri health. The importance of *mātauranga Māori* in managing kauri dieback cannot be overlooked and is essential for us to conserve the long-term health of kauri, the spiritual and cultural tree icon of New Zealand.

Appendix A

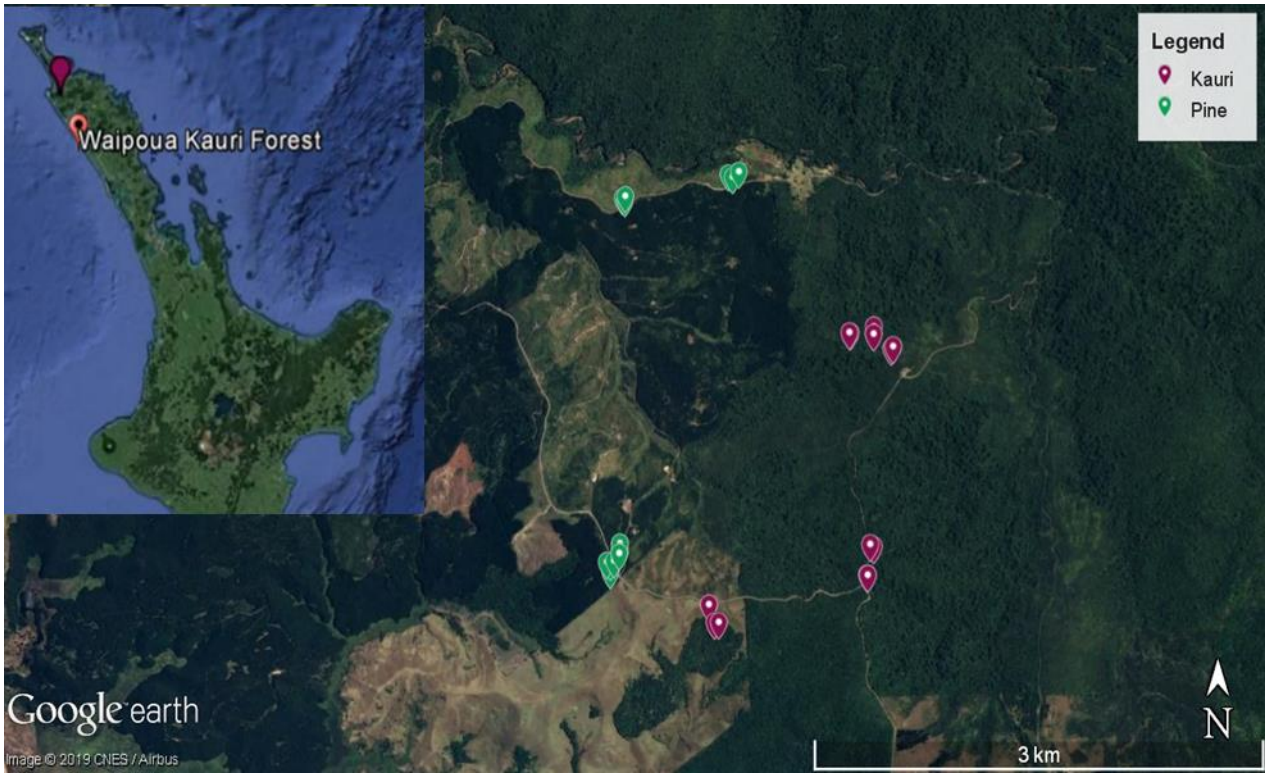

Figure A.1. Locations of soil samples collected from kauri (*Agathis australis*) and pine (*Pinus radiata*) forests across Waipoua Forest (Northland Region, New Zealand).

Table A.1. Results of SIMPER analysis showing the average contributions of fungal OTUs to the Bray Curtis dissimilarity scores. Only OTUs contributing to the cumulative 70% are shown. The '% Contribution' column shows each OTUs contribution to community dissimilarity between kauri and pine soils.

Taxonomic group	Kauri %	Pine %	% Contribution	P-value
Tremellomycetes	20.26	0.53	12.01	<0.001
Apiotrichum gamsii	18.66	0.41	11.10	<0.001
Sistotrema	0.05	8.18	4.95	<0.01
Unidentified	2.28	9.00	4.49	<0.001
Oidiodendron chlamydosporicum	0.31	6.32	3.73	<0.001
Helotiales	1.88	6.77	3.43	>0.05
Penicillium spinulosum	6.29	3.26	3.06	>0.05
Pezizomycotina	4.64	0.30	2.69	<0.001
Sagenomella verticillata	0.08	4.22	2.52	<0.001
Trichoderma spirale	4.39	0.43	2.45	<0.001
Pseudotomentella griseopergamacea	0.02	3.71	2.25	<0.001
Trechispora	3.67	0.20	2.22	>0.05

Hyaloscyphaceae	0.16	3.10	1.82	<0.01
Agaricomycetes	1.29	2.23	1.67	>0.05
Umbelopsis ramanniana	0.07	2.66	1.59	<0.001
Oidiodendron tenuissimum	0.16	2.27	1.32	<0.001
Saccharomycetales	0.12	2.26	1.30	<0.001
Penicillium	0.94	2.38	1.22	<0.001
Hypocreales	0.17	2.06	1.17	<0.001
Amanita muscaria	0.02	1.85	1.12	<0.01
Mortierellaceae	1.87	0.29	1.11	<0.01
Basidiomycota	1.44	0.73	1.10	>0.05
Mortierella humilis	1.94	2.54	1.09	>0.05
Rhizopogon luteolus	0.01	1.78	1.08	>0.05

Table A.2. Results of SIMPER analysis showing the average contributions of bacterial OTUs to the Bray Curtis dissimilarity scores. Only OTUs contributing to the cumulative 70% are shown. The '% Contribution' column shows each OTUs contribution to community dissimilarity between kauri and pine soils.

Taxonomic group	Pine	Kauri	% Contribution	P-value
Enterobacteriaceae	0.28	6.45	8.49	<0.001
Rhodospirillaceae	8.18	7.88	4.84	>0.05
Actinomycetales	5.54	2.53	4.64	<0.001
Actinomycetales	6.32	5.58	4.38	>0.05
Acidobacteriaceae	4.30	2.48	3.17	<0.01
Bradyrhizobiaceae	5.59	5.68	2.57	>0.05
Bacteroidales	1.10	0.97	2.33	>0.05
Acetobacteraceae	3.50	1.95	2.29	<0.001
Rhodoplanes	5.64	5.53	2.02	>0.05
Sinobacteraceae	3.22	2.18	1.78	<0.01
Pseudomonas	0.20	1.32	1.60	<0.001
Lachnospiraceae	0.86	0.68	1.53	>0.05
Candidatus Solibacter	4.15	3.9	1.39	>0.05
Methylocystaceae	2.55	2.06	1.37	>0.05
Burkholderia bryophila	1.75	1.09	1.34	<0.001
Acidobacteria 2	1.72	1.79	1.30	>0.05
Ruminococcaceae	0.53	0.66	1.25	>0.05
Yersinia	0.02	0.92	1.23	<0.001
Acidimicrobiales	1.80	1.08	1.1	<0.001

Granulicella	1.45	1.19	1.17	>0.05
Koribacteraceae	1.90	1.14	1.17	<0.001
Gaiellaceae	1.23	1.03	1.16	>0.05
Burkholderia	1.24	0.61	1.07	<0.001
Pseudomonadaceae	0.12	0.82	1.02	<0.001
Acinetobacter	0.01	0.72	0.98	<0.001
Chitinophagaceae	1.18	1.35	0.97	>0.05
Acidocella	0.78	0.19	0.86	<0.001
Rhizobiales	0.58	1.07	0.78	<0.001
Micromonosporaceae	0.28	0.78	0.74	<0.001
Hyphomonadaceae	0.21	0.60	0.72	<0.001
Solirubrobacterales	1.22	1.23	0.70	>0.05
Rhizobiaceae	0.20	0.60	0.70	<0.001
Sphingomonas wittichii	0.28	0.63	0.69	<0.01
Rhodospirillales	0.25	0.62	0.67	<0.001
Ellin329	1.67	1.40	0.67	>0.05
Acidimicrobiales	0.59	0.76	0.66	>0.05
Burkholderia	0.62	0.16	0.65	<0.001
Mycobacterium celatum	0.69	0.24	0.65	<0.001
Sphingomonas	0.27	0.43	0.61	<0.01
Acidobacteria 6	0.41	0.59	0.60	>0.05
Caulobacteraceae	0.99	0.75	0.57	<0.01
Burkholderiaceae	0.59	0.34	0.52	<0.01
Syntrophobacteraceae	0.86	0.66	0.51	>0.05
Clostridia	0.30	0.20	0.50	>0.05
Chthoniobacteraceae	0.48	0.39	0.50	>0.05
Acinetobacter	0.01	0.37	0.49	<0.001
Oscillospira	0.27	0.24	0.49	>0.05
Caulobacteraceae	0.67	0.34	0.48	<0.001

Table A.3. The number of 16S rRNA and ITS sequencing reads in each DNA extract from kauri and pine soils.

Sample ID	Number of 16s reads	Number of ITS reads
Kauri1	36263	30141
Kauri2	46444	48714
Kauri3	42908	59899
Kauri4	46366	35964
Kauri5	50834	60184
Kauri6	51525	51685
Kauri7	40190	70072
Kauri8	42907	57523
Kauri9	44469	47928
Kauri10	49144	53876
Kauri11	44741	56482
Kauri12	46246	49713
Kauri13	43600	37167
Kauri14	45567	43575
Kauri15	42534	63338
Pine1	54038	59279
Pine2	46464	65581
Pine3	44203	58840
Pine4	51809	60890
Pine5	45163	60211
Pine6	52477	73552
Pine7	46330	70157
Pine8	46615	63183
Pine9	50006	65358
Pine10	50421	65562
Pine11	38195	59888
Pine12	25011	59893
Pine13	31406	65255
Pine14	26318	72510
Pine15	29599	73815

Table A.4. The results of the soil baiting bioassays and real PCR assays used to detect the presence of *P. agathidicida* in kauri and pine soils. Samples were run in triplicate and only samples which had positive Ct values for all three replicates were accepted as testing positive for *P. agathidicida*. *Only one replicate of sample Pine10 had a positive value for *P. agathidicida*, therefore this sample was not considered to be a positive detection.

Sample ID	Soil baiting bioassay	Ct value	Quantity (ng)
Kauri1	Negative	Undetermined	0.00 ± 0.00
Kauri2	Negative	Undetermined	0.00 ± 0.00
Kauri3	Negative	Undetermined	0.00 ± 0.00
Kauri4	Negative	Undetermined	0.00 ± 0.00
Kauri5	Negative	Undetermined	0.00 ± 0.00
Kauri6	Negative	Undetermined	0.00 ± 0.00
Kauri7	Negative	Undetermined	0.00 ± 0.00
Kauri8	Negative	Undetermined	0.00 ± 0.00
Kauri9	Negative	Undetermined	0.00 ± 0.00
Kauri10	Negative	Undetermined	0.00 ± 0.00
Kauri11	Negative	Undetermined	0.00 ± 0.00
Kauri12	Negative	Undetermined	0.00 ± 0.00
Kauri13	Negative	Undetermined	0.00 ± 0.00
Kauri14	Negative	Undetermined	0.00 ± 0.00
Kauri15	Negative	Undetermined	0.00 ± 0.00
Pine1	Negative	Undetermined	0.00 ± 0.00
Pine2	Negative	Undetermined	0.00 ± 0.00
Pine3	Negative	Undetermined	0.00 ± 0.00
Pine4	Negative	Undetermined	0.00 ± 0.00
Pine5	Negative	Undetermined	0.00 ± 0.00
Pine6	Negative	Undetermined	0.00 ± 0.00
Pine7	Negative	Undetermined	0.00 ± 0.00
Pine8	Negative	Undetermined	0.00 ± 0.00
Pine9	Negative	Undetermined	0.00 ± 0.00
Pine10	Negative	36.99*	9.96 E-05
Pine11	Negative	Undetermined	0.00 ± 0.00
Pine12	Negative	Undetermined	0.00 ± 0.00
Pine13	Negative	Undetermined	0.00 ± 0.00
Pine14	Negative	Undetermined	0.00 ± 0.00
Pine15	Negative	Undetermined	0.00 ± 0.00

Appendix B

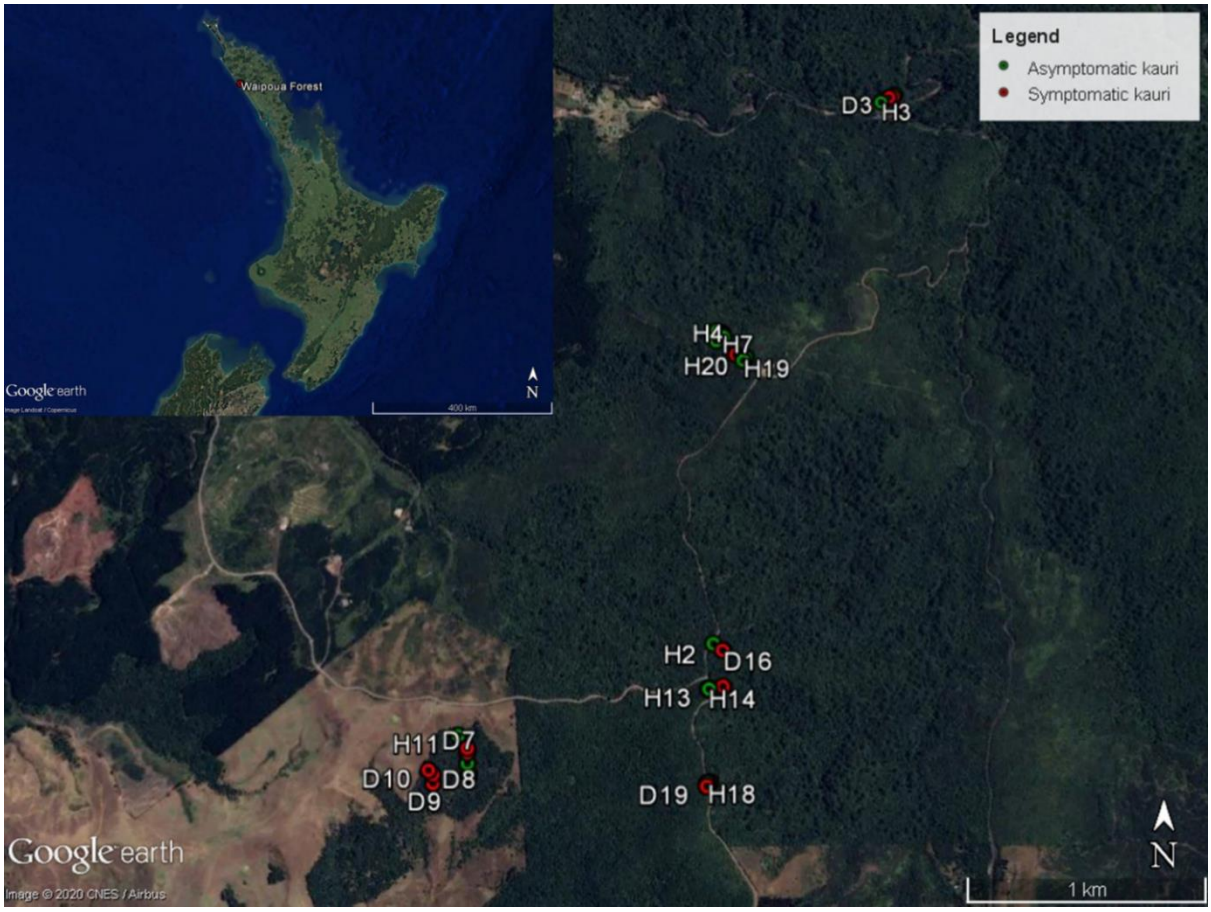

Figure B.1. The locations of the four forest sites that 20 asymptomatic and 20 symptomatic kauri trees were selected for soil sampling in Waipoua Forest (Northland Region, New Zealand).

Supplementary methods B.1. Pathogen bioassays used to detect *Phytophthora agathidicida* in forest soil samples

Field soil samples were detected for the presence or absence of *P. agathidicida* using a soil baiting bioassay (Bellgard et al., 2013) and a real-time PCR assay (McDougal et al., 2014; Than et al., 2013).

For the real-time PCR assays, each soil DNA sample was assayed in triplicate on the StepOnePlus Applied Biosystems real-time PCR instrument (Thermo Fisher Scientific, Massachusetts USA). Each 15µl reaction consisted of 1µl DNA, 0.525µl of each forward and reverse primer, 0.24µl probe (Table B.1, Appendix B), 7.5µl TaqMan Environmental master mix (Thermo Fisher Scientific, Massachusetts USA) and 5.5µl nuclease free water (Invitrogen, Massachusetts USA). Thermocycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 61°C for 60 s. A standard curve was

prepared for each assay using a 5-fold 1:10 serial dilution of a *P. agathidicida* DNA that ranged from 2.8ng/µl to 0.00028ng/µl. Using the prepared standard curve, the 'absolute quantification' method was used to determine the concentration of *P. agathidicida* DNA (or cDNA) in each sample, with these calculations being performed by the StepOnePlus real-time PCR instrument.

This study used the standard operating soil baiting protocol developed for *P. agathidicida* (Bellgard et al., 2013). For each soil sample, 175g of soil was air dried for 3 days. On day four, soils were incubated with distilled water until moist but not soaked. On day 5, five lupin seeds per soil sample were set up to germinate using vermiculate and water, however these were set up in triplicate to account for failed germinations. On day 8, soil samples were flooded with 500ml of distilled water and 5 germinated lupins were set afloat on polystyrene sheets so that they were submerged but not touching the soil. Additionally, five cedar needles per sample were also set afloat. On day 10, lupin and cedar needle baits were harvested, plated onto *Phytophthora* selective CRNH media and then incubated at 22^{o}C in darkness (Herewini et al., 2018). For harvesting, a 1 cm end segment of each bait was surface sterilised in ethanol for 30 seconds followed by two 30 second rinses in water. After 2 to 5 days of growth on selective media, *Phytophthora* cultures were sub cultured onto CAD media (Herewini et al., 2018) and incubated for up to 1 week prior to morphological identification. Key morphological features used to distinguish *P. agathidicida* from other *Phytopthora* pathogens included: globose oogonia with mildly stipulate oogonium ornamentation and amphigynous, sub-globose antheridia; large mean oospore diameter (~32µm); absence of chlamydospores; simple vegetative hyphal growth; and uniform and lightly aerial colony morphology on agar (Weir et al., 2015).

Cultures which could not be identified based on morphology were identified by Sanger sequencing of the ITS gene region. Mycelium was harvested from cultures and DNA extracted using a DNeasy Plant Extraction kit (Qiagen, Germany). Following DNA extraction, primers ITS4 and ITS6 were used to amplify the ITS gene region (Cooke et al., 2000). Each 25µl reaction consisted of 1µl DNA, 0.5µl of each forward and reverse primer, 0.2µl MyTaq DNA polymerase and 5µl MyTaq reaction buffer (Bioline, UK) and 17.8µl nuclease free water (Invitrogen, Massachusetts USA). PCR thermocycling conditions were 94^{o}C for 1 min followed by 38 cycles of 94^{o}C for 45 seconds, 52^{o}C for 45 seconds, 72^{o}C for 45 seconds and a final extension of 72^{o}C for 7 minutes. PCR products were ran on 1% agarose gel at 100V for 15 minutes to confirm successful amplification of the desired band size. Successful PCR products were cleaned using the AxyPrep magnetic bead purification kit (Axygen Scientific, California USA) and sequenced at the Lincoln University Sequencing Facility, New Zealand. Following sequencing, forward and reverse reads were aligned and low quality ends trimmed using

Unipro UGENE software (Okonechnikov et al., 2012). Reads were identified to species level using BLAST (National Centre for Biotechnology Information, NCBI).

Table B.1. The forward and reverse primers and TaqMan probe used to detect *P. agathidicida* using real-time PCR, as developed by Than et al. (2013) and edited by McDougal et al. (2014).

Primer name	Oligo sequence
ITS_PTA_F2	'5- AACCAATAGTTGGGGGCGA -3'
ITS_PTA_R3	'5- CTCGCCATGATAGAGCTCGTC -3'
ITS_PTA_probe	'5- /56-FAM/ AGCCAAAGCCAGCAGCCG /3BHQ_1/ -3'

Table B.2. The results of the soil baiting bioassays and TaqMan real-time PCR used to detect the presence of *P. agathidicida* in soil samples. Results of the TaqMan real-time PCR are reported with their cycling threshold values (mean average ± standard deviation), R^2 values and cycling efficiency.

Tree ID	Soil baiting bioassay	TaqMan qPCR	Cycle threshold value	R2	Efficiency (%)
SYMP1	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP2	Positive	Positive	35.24 ± 1.20	0.96	90
SYMP3	Positive	Positive	30.04 ± 0.76	0.96	90
SYMP4	Negative	Positive	36.30 ± 1.0	0.96	90
SYMP5	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP6	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP7	Positive	Negative	0.00 ± 0.00	0.96	90
SYMP8	Positive	Positive	30.34 ± 0.05	0.96	90
SYMP9	Positive	Positive	35.6 ± 1.10	0.96	90
SYMP10	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP11	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP12	Positive	Negative	0.00 ± 0.00	0.96	90
SYMP13	Negative	Positive	35.62 ± 1.17	0.96	90
SYMP14	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP15	Negative	Positive	30.82 ± 0.12	0.96	90
SYMP16	Positive	Negative	0.00 ± 0.00	0.96	90
SYMP17	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP18	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP19	Negative	Negative	0.00 ± 0.00	0.96	90
SYMP20	Positive	Negative	0.00 ± 0.00	0.96	90
ASYMP1	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP2	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP3	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP4	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP5	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP6	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP7	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP8	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP9	Negative	Negative	0.00 ± 0.00	0.99	92

ASYMP10	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP11	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP12	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP13	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP14	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP15	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP16	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP17	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP18	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP19	Negative	Negative	0.00 ± 0.00	0.99	92
ASYMP20	Negative	Negative	0.00 ± 0.00	0.99	92

Combining the results of the TaqMan real-time PCR and soil baiting bioassay, 11 out of 20 symptomatic soil samples tested positive for *P. agathidicida.* No asymptomatic soil samples tested positive for *P. agathidicida* (Table S2). All 11 symptomatic soil samples which tested positive for *P. agathidicida*, and 4 other symptomatic soil samples, were selected to be sent off for high throughput sequencing. 5 positive symptomatic soil samples were sent off for GeoChip 5S microarray analysis. The relatively low recoveries of *P. agathidicida* in symptomatic soil samples may be explained by several factors. Extensive surveys have identified that even surrounding heavily infected kauri trees, recovery of *P. agathidicida* from soil samples is highly variable due to their patchy and inconsistent environmental distribution (Bellgard et al., 2013). Additionally, the stage of disease can cause fluctuations in soil inoculum levels. Commonly, once tree crown symptoms are evident, soil inoculum levels decrease as the pathogen advances within the root system. During this stage there is also commonly an increase in abundance of secondary pathogenic and saprophytic fungi which can mask the primary causes of disease (Jung et al., 2018).

Table B.3. The mean average ± standard deviation values for the alpha diversity values calculated for carbon and nitrogen cycling genes in asymptomatic and symptomatic soils.

Alpha diversity metric	Asymptomatic	Symptomatic	T test results
Number of detected probes	23648 ± 788	22362 ± 1285	t = 1.71, p-value = 0.13
Shannon diversity	9.03 ± 0.05	8.87 ± 0.12	t = 2.43, p-value = 0.05
Pielou's evenness	0.90 ± 0.00	0.89 ± 0.01	t = 2.38, p-value = 0.07

Table B.4. Results of DESeq2 analysis showing the fungal OTUs with a significantly different relative abundance between asymptomatic and symptomatic kauri soils. Positive 'log2 Fold Change' values are fungal OTUs found significantly higher in asymptomatic soil and negative values are those significantly higher in symptomatic soil.

Taxa ID of fungal OTU	log2 Fold Change	p-value	Taxa ID of fungal OTU	log2 Fold Change	p-value
Cystolepiota	-5.893	0.0000	Oidiodendron	-2.930	0.0074
Archaeorhizomyces	-4.522	0.0000	Nectriaceae	-3.410	0.0000
Archaeorhizomyces	-3.654	0.0047	Nectriaceae	2.487	0.0002
Aspergillus flavus	-2.359	0.0010	Rhodocollybia	-4.954	0.0000
Penicillium	1.626	0.0064	Tolypocladium	-3.206	0.0000
Penicillium	2.134	0.0036	Tolypocladium	-2.287	0.0022
Penicillium	2.217	0.0000	Tolypocladium	-1.915	0.0009
Penicillium herquei	2.323	0.0064	Phacidiaceae	-2.373	0.0005
Penicillium	2.348	0.0005	Barnettozyma californica	2.647	0.0014
Penicillium	2.557	0.0002	Urnula	-2.521	0.0024
Penicillium	2.766	0.0050	Hyphodontia	-3.275	0.0057
Penicillium	2.956	0.0033	Schizoporaceae	-4.364	0.0000
Penicillium	3.106	0.0044	Schizoporaceae	-2.604	0.0006
Penicillium	3.115	0.0002	Serendipita	-3.503	0.0029
Penicillium	3.575	0.0000	Serendipita	-3.241	0.0040
Aspergillaceae	-1.534	0.0036	Serendipita	-3.135	0.0000
Aspergillaceae	1.914	0.0045	Galerina	-5.479	0.0000
Auricularia	3.242	0.0072	Phaeoacremonium	-3.773	0.0000
Backusella lamprospora	3.096	0.0021	Delicatula	-2.594	0.0017
Botryobasidium	-3.035	0.0027	Mycena	-3.306	0.0017
Chaetosphaeria	-4.293	0.0027	Mycena	-2.944	0.0006
Chaetosphaeria	-3.680	0.0010	Mycena	-2.665	0.0024
Clavaria	-3.345	0.0001	Tricholomataceae	-4.070	0.0002

Clavaria	-3.229	0.0000	Tricholomataceae	-3.075	0.0009
Clavulinopsis	-5.233	0.0000	Tricholomataceae	-2.921	0.0000
Clavulinopsis	-4.802	0.0001	Tricholomataceae	3.864	0.0043
Clavulinopsis	-3.224	0.0000	Spencermartinsiella	-4.995	0.0000
Clavulinopsis	-2.946	0.0031	Apiotrichum	2.233	0.0012
Clavariaceae	-4.589	0.0003	Apiotrichum	2.464	0.0004
Clavariaceae	-2.738	0.0051	Apiotrichum	2.741	0.0012
Clavariaceae	-4.446	0.0000	Apiotrichum	3.171	0.0006
Clavariaceae	-4.360	0.0000	Trichosporonaceae	2.119	0.0038
Clavariaceae	-4.061	0.0000	Trichosporonaceae	2.345	0.0012
Clavariaceae	-4.006	0.0002	Trichosporonaceae	2.693	0.0002
Clavariaceae	-3.525	0.0003	Trichosporonaceae	2.717	0.0023
Clavariaceae	-3.286	0.0004	Trichosporonaceae	2.883	0.0013
Clavariaceae	-3.256	0.0002	Trichosporonaceae	2.908	0.0000
Clavariaceae	-3.092	0.0040	Trichosporonaceae	3.046	0.0003
Clavariaceae	-3.087	0.0054	Trichosporonaceae	3.134	0.0000
Clavariaceae	-3.066	0.0009	Trichosporonaceae	3.299	0.0000
Clavariaceae	-2.990	0.0015	Trichosporonaceae	3.966	0.0011
Clavariaceae	-2.894	0.0007	Umbelopsis	-2.289	0.0056
Clavariaceae	-2.863	0.0001	Chaetothyriales	-6.385	0.0000
Clavariaceae	-2.343	0.0018	Saccharomycetales	-5.637	0.0000
Clavicipitaceae	2.290	0.0010	Saccharomycetales	-5.269	0.0001
Absidia glauca	2.753	0.0066	Saccharomycetales	-5.212	0.0001
Meliniomyces	-3.374	0.0000	Hymenochaetales	-4.932	0.0043
Leohumicola	-4.420	0.0014	Agaricomycetes	-4.506	0.0000
Leohumicola	-2.108	0.0049	Helotiales	-4.492	0.0006
Leohumicola	-1.943	0.0068	Trechisporales	-3.759	0.0028
Leohumicola	-1.622	0.0079	Saccharomycetales	-3.751	0.0004
Helotiales_fam_Incertae_sedis	-3.711	0.0008	Helotiales	-3.440	0.0036

Cladophialophora	-1.899	0.0024	Lecanoromycetes	-3.412	0.0005
Herpotrichiellaceae	-5.021	0.0000	Rozellomycotina_cls_Incertae_sedis (GS11)	-3.339	0.0001
Herpotrichiellaceae	-4.934	0.0000	unidentified	-3.322	0.0023
Herpotrichiellaceae	-4.676	0.0000	Helotiales	-3.318	0.0009
Herpotrichiellaceae	-4.091	0.0000	Eurotiales	-3.309	0.0002
Herpotrichiellaceae	-3.591	0.0018	Helotiales	-3.245	0.0009
Herpotrichiellaceae	-3.557	0.0001	Saccharomycetales	-3.173	0.0029
Herpotrichiellaceae	-3.489	0.0006	Hypocreales	-3.157	0.0003
Herpotrichiellaceae	-3.284	0.0000	Helotiales	-2.937	0.0008
Herpotrichiellaceae	-3.274	0.0004	Auriculariales	-2.870	0.0022
Herpotrichiellaceae	-3.157	0.0000	Rozellomycotina_cls_Incertae_sedis (GS14)	-2.829	0.0076
Herpotrichiellaceae	-3.135	0.0000	Chytridiales	-2.743	0.0050
Herpotrichiellaceae	-3.036	0.0004	Chaetothyriales	-2.697	0.0065
Herpotrichiellaceae	-2.770	0.0021	unidentified	-2.455	0.0032
Herpotrichiellaceae	-2.762	0.0000	Helotiales	-2.384	0.0016
Herpotrichiellaceae	-2.739	0.0002	Helotiales	-2.070	0.0058
Herpotrichiellaceae	-2.625	0.0004	Venturiales	-3.376	0.0000
Herpotrichiellaceae	-2.406	0.0094	Venturiales	-2.847	0.0020
Herpotrichiellaceae	-2.353	0.0032	Venturiales	-2.519	0.0002
Herpotrichiellaceae	-4.492	0.0000	Venturiales	-2.068	0.0071
Herpotrichiellaceae	-3.715	0.0012	Venturiales	-1.923	0.0058
Herpotrichiellaceae	-3.471	0.0000	Xenasmatella	-2.603	0.0010
Herpotrichiellaceae	-3.377	0.0001	Cantharellales	-5.123	0.0003
Herpotrichiellaceae	-2.769	0.0028	Ascomycota	-4.602	0.0000
Herpotrichiellaceae	-2.747	0.0000	Ascomycota	-4.524	0.0000
Herpotrichiellaceae	-2.738	0.0000	Leotiomycetes	-4.246	0.0009
Herpotrichiellaceae	-2.475	0.0001	Auriculariales	-4.245	0.0001
Hyaloscypha	-2.936	0.0012	Agaricomycetes	-4.204	0.0000
Hyaloscyphaceae	-3.969	0.0000	Agaricales	-4.007	0.0000

Hyaloscyphaceae	-3.611	0.0001	Helotiales	-3.807	0.0030
Luellia	-5.794	0.0000	Agaricomycetes	-3.770	0.0020
Trechispora	-5.340	0.0000	Chaetothyriales	-3.623	0.0000
Trechispora	-5.109	0.0033	Ascomycota	-3.562	0.0016
Trechispora	-5.108	0.0000	Helotiales	-3.520	0.0000
Trechispora	-5.097	0.0000	Helotiales	-3.519	0.0014
Trechispora	-4.739	0.0000	Helotiales	-3.485	0.0000
Trechispora	-4.582	0.0030	Chaetothyriales	-3.442	0.0014
Trechispora	-4.393	0.0000	Chaetothyriales	-3.389	0.0010
Trechispora	-3.241	0.0031	Helotiales	-3.335	0.0098
Trechispora	-2.904	0.0001	Agaricales	-3.251	0.0005
Trechispora	-2.849	0.0098	Helotiales	-3.174	0.0011
Trechispora	-2.218	0.0074	Ascomycota	-3.166	0.0000
Hydnodontaceae	-3.013	0.0004	Leotiomycetes	-2.979	0.0014
Cuphophyllus	-5.475	0.0000	Dothideomycetes	-2.957	0.0004
Cuphophyllus	-4.823	0.0000	Helotiales	-2.956	0.0092
Cuphophyllus	-4.542	0.0046	Helotiales	-2.923	0.0100
Cuphophyllus	-4.352	0.0001	Dothideomycetes	-2.834	0.0062
Cuphophyllus	-3.614	0.0098	Helotiales	-2.825	0.0000
Humidicutis	-2.426	0.0023	Ascomycota	-2.801	0.0000
Hygrocybe	-6.750	0.0000	Helotiales	-2.793	0.0014
Hygrocybe	-4.843	0.0022	Sordariomycetes	-2.684	0.0009
Hygrophoraceae	-2.883	0.0005	Venturiales	-2.647	0.0010
Hygrophoraceae	-2.747	0.0003	Helotiales	-2.521	0.0020
Hyphoderma	-4.125	0.0019	Helotiales	-2.521	0.0013
Hyphoderma	-3.869	0.0000	Helotiales	-2.504	0.0040
Trichoderma	-3.162	0.0011	Ascomycota	-2.378	0.0010
Trichoderma	2.052	0.0057	Ascomycota	-2.370	0.0065
Trichoderma	2.162	0.0012	Ascomycota	-2.272	0.0065

Trichoderma	3.120	0.0000	Helotiales	-2.215	0.0029
Trichoderma	3.473	0.0000	Helotiales	-2.141	0.0038
Leotiaceae	-1.792	0.0028	Hypocreales	-2.140	0.0086
Mortierella	-3.778	0.0000	Helotiales	-1.824	0.0045
Mortierella	-2.202	0.0009	Ascomycota	-1.814	0.0047
Mortierella	-2.102	0.0016	Helotiales	-1.813	0.0015
Mortierella	-1.979	0.0067	Agaricomycetes	-1.800	0.0033
Mortierella	-1.741	0.0000	Cystofilobasidiales	2.814	0.0028
Oidiodendron	-3.802	0.0009	Xylariales	4.967	0.0006

Table B.5. Results of DESeq2 analysis showing the bacterial OTUs with a significantly different relative abundance between asymptomatic and symptomatic kauri soils. Positive 'log2 Fold Change' values are bacterial OTUs found significantly higher in asymptomatic soil and negative values are those significantly higher in symptomatic soil.

Taxa ID of bacterial OTU	log2 Fold Change	p-value	Taxa ID of bacterial OTU	log2 Fold Change	p-value
Acinetobacter	5.169	0.0000	Hyphomicrobiaceae	1.774	0.0027
Acinetobacter	5.133	0.0000	Rhodoplanes	1.751	0.0024
Enterobacteriaceae	4.951	0.0000	Ellin6067	1.695	0.0030
Leuconostocaceae	4.711	0.0002	Chthoniobacteraceae (DA101)	1.679	0.0061
Enterobacteriaceae	4.687	0.0000	Ellin6076	1.642	0.0083
Enterobacteriaceae	4.641	0.0000	Rhizobiaceae	1.623	0.0003
Enterobacteriaceae	4.631	0.0000	Nocardioides	1.612	0.0031
Enterobacteriaceae	4.592	0.0000	Steroidobacter	1.600	0.0088
Enterobacteriaceae	4.574	0.0000	Hyphomonadaceae	1.597	0.0091
Enterobacteriaceae	4.415	0.0000	Gammaproteobacteria (HTCC2090)	1.531	0.0011
Enterobacteriaceae	4.382	0.0005	Phenylobacterium	1.519	0.0000
Enterobacteriaceae	4.327	0.0000	Candidatus Solibacter	1.513	0.0013
Enterobacteriaceae	4.263	0.0000	Comamonadaceae	1.512	0.0028
Enterobacteriaceae	4.248	0.0000	Comamonadaceae	1.509	0.0018
Enterobacteriaceae	4.212	0.0000	Hyphomicrobiaceae	1.469	0.0070
Enterobacteriaceae	4.155	0.0000	Streptomyces	1.434	0.0053
Acinetobacter	4.052	0.0000	Comamonadaceae	1.426	0.0058
Acinetobacter	3.936	0.0006	Acidimicrobiales (EB1018)	1.353	0.0100
Pseudomonas	3.936	0.0000	Hyphomicrobiaceae	1.352	0.0045
Enterobacteriaceae	3.881	0.0000	Comamonadaceae	1.347	0.0009
Enterobacteriaceae	3.835	0.0003	Mycobacterium	1.343	0.0046
Pseudomonas	3.769	0.0000	Acidimicrobiales (C111)	1.300	0.0082
Actinobacteria (0319-7L14)	3.716	0.0002	Comamonadaceae	1.295	0.0004
Enterobacteriaceae	3.715	0.0000	Rhodoplanes	1.284	0.0058

Enterobacteriaceae	3.646	0.0023	Rhizobiaceae	1.263	0.0090
Enterobacteriaceae	3.639	0.0002	Sphingomonas	1.197	0.0097
Rhizobiaceae	3.624	0.0027	Candidatus Solibacter	1.183	0.0058
Enterobacteriaceae	3.608	0.0014	Bradyrhizobium	1.132	0.0079
Enterobacteriaceae	3.595	0.0001	Rhodospirillaceae	1.012	0.0056
Flavobacterium	3.587	0.0011	Solibacterales	0.971	0.0100
Pseudomonas	3.585	0.0003	Comamonadaceae	0.766	0.0077
Chloracidobacteria (DS-100)	3.580	0.0004	Ellin6513	-0.748	0.0098
Enterobacteriaceae	3.542	0.0001	Polyangiaceae	-1.050	0.0045
Pseudomonas	3.521	0.0003	Syntrophobacteraceae	-1.052	0.0069
Oxalobacteraceae	3.364	0.0003	Myxococcales	-1.065	0.0090
Microbacteriaceae	3.314	0.0069	Alphaproteobacteria	-1.114	0.0015
Enterobacteriaceae	3.308	0.0013	Solibacterales	-1.126	0.0045
Enterobacteriaceae	3.278	0.0077	Ellin6513	-1.277	0.0040
Pseudomonas	3.250	0.0000	Caulobacteraceae	-1.289	0.0066
Erwinia	3.179	0.0000	Actinomycetales	-1.301	0.0007
Enterobacteriaceae	3.132	0.0095	Ellin6513	-1.338	0.0083
Pseudomonas	3.128	0.0009	Acidobacteriaceae	-1.340	0.0008
Acidobacteria-6 (iii1-15)	3.101	0.0081	Solirubrobacterales	-1.368	0.0015
Chloracidobacteria (RB41)	3.077	0.0013	Methylocystaceae	-1.383	0.0064
Sphingomonadaceae	3.040	0.0030	Koribacteraceae	-1.391	0.0057
Pseudomonas	2.954	0.0003	Acidimicrobiales	-1.480	0.0085
Sphingomonas wittichii	2.931	0.0003	Rhodospirillaceae	-1.485	0.0011
Pseudomonas	2.930	0.0000	Conexibacteraceae	-1.504	0.0014
Bradyrhizobiaceae	2.927	0.0011	Acidocella	-1.509	0.0021
Acinetobacter	2.907	0.0083	Sinobacteraceae	-1.524	0.0042
Chthoniobacteraceae (DA101)	2.853	0.0052	Actinomycetales	-1.533	0.0007
Sphingomonadaceae	2.853	0.0018	Candidatus Solibacter	-1.544	0.0087
Microbacteriaceae	2.842	0.0010	Rhodospirillaceae	-1.590	0.0008

Micromonosporaceae	2.832	0.0013	Koribacteraceae	-1.644	0.0011
Pseudomonas	2.798	0.0000	Acidobacteriaceae	-1.667	0.0098
Pseudomonas	2.770	0.0047	Acidobacteriaceae	-1.669	0.0037
Bacillus	2.657	0.0018	Ellin6513	-1.676	0.0040
Solirubrobacterales	2.643	0.0019	Actinomycetales	-1.695	0.0031
Hyphomonadaceae	2.616	0.0069	Deltaproteobacteria (JTB36)	-1.708	0.0083
Pseudomonas	2.517	0.0051	Burkholderia	-1.718	0.0086
Pseudonocardiaceae	2.513	0.0074	Ellin6513	-1.740	0.0063
Mycobacterium	2.506	0.0088	Acetobacteraceae	-1.809	0.0087
Rhodobacteraceae	2.504	0.0042	Acidobacteriaceae	-1.832	0.0100
Demequina	2.491	0.0051	Ellin6513	-1.838	0.0029
Betaproteobacteria (SC-I-84)	2.490	0.0069	Koribacteraceae	-1.881	0.0024
Hyphomicrobium	2.486	0.0086	Myxococcales	-1.897	0.0064
Pseudomonas	2.461	0.0097	Acidobacteriaceae	-1.904	0.0023
Cellulomonas	2.416	0.0006	Telmatospirillum	-1.944	0.0095
Pseudomonas	2.389	0.0027	Koribacteraceae	-1.954	0.0028
Lactobacillus	2.377	0.0097	Ellin6513	-1.961	0.0003
Amaricoccus	2.347	0.0069	WPS-2	-1.984	0.0050
Sphingobacteriaceae	2.314	0.0087	Rhodospirillaceae	-2.001	0.0044
Sphingomonadaceae	2.305	0.0004	Acidimicrobiales	-2.020	0.0069
Paenibacillus	2.254	0.0067	Acidimicrobiales	-2.022	0.0010
Pseudomonas	2.235	0.0054	Rhodoplanes	-2.032	0.0027
Enterobacteriaceae	2.212	0.0100	Acidimicrobiales	-2.039	0.0036
Sphingomonadaceae	2.206	0.0011	Pedosphaerales (auto67_4W)	-2.067	0.0018
Xanthomonadaceae	2.206	0.0003	Ellin6513	-2.073	0.0022
Microbacteriaceae	2.202	0.0001	Pedosphaerales	-2.081	0.0035
Conexibacteraceae	2.194	0.0051	Pedosphaerales	-2.084	0.0000
Rhodococcus	2.158	0.0098	Acidimicrobiales	-2.116	0.0026
Chitinophagaceae	2.154	0.0064	Acidobacteria (TM1)	-2.164	0.0050

Koribacteraceae	2.143	0.0045	WPS-2	-2.180	0.0083
Sphingomonadaceae	2.140	0.0040	Pedosphaerales (auto67_4W)	-2.187	0.0004
Caulobacteraceae	2.108	0.0008	Mycobacterium	-2.191	0.0021
Microbacteriaceae	2.103	0.0070	Methylacidiphilae (S-BQ2-57)	-2.271	0.0090
Sphingomonas wittichii	2.077	0.0003	Solirubrobacterales	-2.281	0.0015
Pseudomonas	2.018	0.0028	Koribacteraceae	-2.303	0.0020
Cellulomonas	1.987	0.0058	Koribacteraceae	-2.363	0.0043
Asticcacaulis	1.945	0.0046	Actinomycetales	-2.417	0.0083
Comamonadaceae	1.908	0.0036	Syntrophobacteraceae	-2.511	0.0013
Candidatus Solibacter	1.881	0.0011	Solirubrobacterales	-2.667	0.0019
Micromonosporaceae	1.872	0.0017	Bacteroidetes	-2.865	0.0001
Candidatus Solibacter	1.867	0.0093	Opitutus	-2.945	0.0071
Rhizobiales	1.853	0.0058	Koribacteraceae	-2.951	0.0008
Rhodospirillaceae	1.841	0.0021	Ellin6513	-2.977	0.0022
Acidimicrobiales (C111)	1.839	0.0100	Koribacteraceae	-3.005	0.0059
Caulobacteraceae	1.838	0.0014	Betaproteobacteria	-3.235	0.0003
Mesorhizobium	1.785	0.0068	Clostridiaceae	-3.242	0.0010

Table B.6. Results of DESeq2 analysis showing the carbon and nitrogen cycling genes with a significantly different relative abundance between asymptomatic and symptomatic kauri soils. Positive 'log2 Fold Change' values (Lfc) are genes found significantly higher in asymptomatic soil and negative values are those significantly higher in symptomatic soil.

Gene function	Gene name	log2 Fold Change	P-value	Gene function	Gene name	log2 Fold Change	P-value
Pectin deg.	RgaE	25.66	0.000	Calvin cycle	pgk	-0.94	0.007
Chitin deg.	acetylglucosaminidase	25.57	0.000	Denitrification	nosz	-0.94	0.008
Denitrification	nosz	25.53	0.000	Assim. N red.	nasa	-0.94	0.004
Denitrification	narg	25.4	0.000	Starch deg.	amyA	-0.94	0.004
Tannin deg.	tannase_Cdeg	25.36	0.000	Denitrification	nirk	-0.94	0.000
Cellulose deg.	cellobiase	25.33	0.000	Denitrification	narg	-0.94	0.008
Calvin cycle	rubisco	25.3	0.000	Reductive acetyl CCoA pathway	fthfs	-0.94	0.006
Cellulose deg.	cellobiase	25.26	0.000	Reductive acetyl CCoA pathway	fthfs	-0.94	0.008
Heparin deg.	heparinase	25.26	0.000	Starch deg.	amyA	-0.95	0.003
Nitro. fix.	nifh	25.24	0.000	Denitrification	narg	-0.95	0.010
Pectin deg.	rgh	25.22	0.000	Bacterial Microcompartments	ccmL	-0.95	0.007
Starch deg.	amyA	25.15	0.000	Starch deg.	cda	-0.96	0.009
Phospholipid deg.	phospholipase_C_fungi	25.1	0.000	Nitro. fix.	nifh	-0.96	0.005
Starch deg.	amyA	25.07	0.000	Calvin cycle	FBP_aldolase	-0.96	0.009
Hemicellulose deg.	xyla	25.07	0.000	Denitrification	nirk	-0.96	0.000
Pectin deg.	RgaE	25.06	0.000	Cellulose deg.	exoglucanase	-0.96	0.008
Cellulose deg.	endoglucanase	25.02	0.000	Nitro. fix.	nifh	-0.96	0.002
Red. acetyl CCoA pathway	fthfs	24.96	0.000	Starch deg.	amyA	-0.96	0.000
Starch deg.	amyA	24.92	0.000	Starch deg.	amyA	-0.96	0.004
Phospholipid deg.	phospholipase_A2_fungi	24.92	0.000	Pectin deg.	pme	-0.97	0.007
Ammonification	gdh	24.91	0.000	Starch deg.	amyA	-0.97	0.006
Nitro. fix.	nifh	24.91	0.000	Denitrification	nosz	-0.97	0.006

Denitrification	nirs	24.89	0.000	Calvin cycle	rubisco	-0.97	0.001
Chitin deg.	chitinase	24.85	0.000	Starch deg.	amyA	-0.97	0.006
Starch deg.	amyA	24.83	0.000	Denitrification	nirk	-0.97	0.008
Starch deg.	amyA	24.82	0.000	Denitrification	nosz	-0.97	0.003
Hemicellulose deg.	ara	24.82	0.000	Starch deg.	amyA	-0.98	0.001
Denitrification	nosz	24.81	0.000	Starch deg.	amyA	-0.98	0.005
Nitro. fix.	nifh	24.8	0.000	Calvin cycle	TIM	-0.98	0.002
Calvin cycle	PRI	24.8	0.000	Starch deg.	glucoamylase	-0.98	0.008
Calvin cycle	pgk	24.78	0.000	Denitrification	norb	-0.98	0.005
Calvin cycle	rubisco	24.78	0.000	Pectin deg.	RgaE	-0.98	0.000
Denitrification	nirs	24.77	0.000	Starch deg.	pula	-0.98	0.007
Hemicellulose deg.	ara	24.74	0.000	Cellulose deg.	endoglucanase	-0.98	0.004
Calvin cycle	FBPase	24.71	0.000	Starch deg.	amyA	-0.99	0.001
Calvin cycle	rubisco	24.7	0.000	Nitro. fix.	nifh	-0.99	0.008
Starch deg.	glucoamylase	24.69	0.000	Chitin deg.	acetylglucosaminidase	-0.99	0.001
Starch deg.	amyA	24.69	0.000	Starch deg.	amyA	-0.99	0.003
Denitrification	nosz	24.69	0.000	Calvin cycle	GAPDH_Calvin	-1	0.010
Starch deg.	amyA	24.68	0.000	Starch deg.	amyA	-1	0.001
Hemicellulose deg.	ara	24.68	0.000	Pectin deg.	rgl	-1	0.003
Calvin cycle	tktA	24.67	0.000	Dissim. N fix.	napa	-1	0.002
Calvin cycle	GAPDH_Calvin	24.67	0.000	Chitin deg.	exochitinase	-1.01	0.003
Cellulose deg.	endoglucanase	24.67	0.000	Starch deg.	cda	-1.01	0.003
Denitrification	narg	24.65	0.000	Starch deg.	amyA	-1.01	0.000
Starch deg.	cda	24.64	0.000	Calvin cycle	rubisco	-1.01	0.009
Reductive acetyl CCoA pathway	fthfs	24.64	0.000	Hemicellulose deg.	ara	-1.02	0.009
Starch deg.	cda	24.63	0.000	Reductive acetyl CCoA pathway	fthfs	-1.02	0.001
Lignin deg.	mnp	24.62	0.000	Assim. N red.	nirb	-1.02	0.007
Calvin cycle	pgk	24.62	0.000	Pectin deg.	pectinase (pectate_lyase)	-1.02	0.005

Starch deg.	cda	24.61	0.000	Chitin deg.	endochitinase	-1.02	0.001
Chitin deg.	chitinase	24.6	0.000	Terpenes deg.	limeh	-1.03	0.000
Denitrification	nirk	24.6	0.000	Denitrification	narg	-1.03	0.004
Starch deg.	amyA	24.6	0.000	Ammonification	urec	-1.03	0.010
Nitro. fix.	nifh	24.58	0.000	Starch deg.	glucoamylase	-1.03	0.008
Chitin deg.	chitinase	24.55	0.000	Denitrification	nirk	-1.03	0.000
Denitrification	narg	24.55	0.000	Calvin cycle	PRK	-1.03	0.003
Denitrification	nosz	24.54	0.000	Denitrification	nirk	-1.04	0.000
Starch deg.	amyA	24.52	0.000	Glucose deg.	Glucose_oxidase_fungi	-1.04	0.000
Denitrification	narg	24.51	0.000	Starch deg.	amyA	-1.05	0.006
Methane oxidation	pmoa	24.51	0.000	Nitro. fix.	nifh	-1.05	0.001
Denitrification	nirs	24.51	0.000	Starch deg.	amyA	-1.05	0.001
Lignin	glx	24.46	0.000	Denitrification	nirs	-1.05	0.008
Calvin cycle	FBPase	24.45	0.000	Pectin deg.	rgl	-1.06	0.001
Starch deg.	amyA	24.44	0.000	Starch deg.	amyA	-1.07	0.001
Chitin deg.	chitinase	24.44	0.000	Calvin cycle	FBP_aldolase	-1.07	0.002
Bacterial Microcompartments	ccmL	24.41	0.000	N Assimilation	nitrate_reductase	-1.07	0.003
Calvin cycle	TIM	24.39	0.000	Hemicellulose deg.	xylanase	-1.07	0.008
Calvin cycle	pgk	24.39	0.000	Chitin deg.	chitinase	-1.07	0.004
Lignin deg.	phenol_oxidase	24.38	0.000	Assim. N red.	nasa	-1.07	0.002
Phospholipid deg.	phospholipase_C_fungi	24.38	0.000	Starch deg.	amyA	-1.08	0.004
Reductive tricarboxylic acid cycle	oorA	24.38	0.000	Pectin deg.	pme	-1.09	0.003
Starch deg.	amyA	24.37	0.000	Cutin deg.	cutinase	-1.1	0.002
Reductive tricarboxylic acid cycle	ccl	24.35	0.000	Calvin cycle	tktA	-1.12	0.000
Denitrification	nirs	24.34	0.000	Pectin deg.	rgl	-1.12	0.001
Cellulose deg.	cellobiase	24.34	0.000	Pectin deg.	pectinase (pectate_lyase)	-1.13	0.006
Chitin deg.	chitinase	24.19	0.000	Hemicellulose deg.	ara	-1.13	0.005
Calvin cycle	tktA	24.16	0.000	Terpenes deg.	cdh	-1.14	0.002
Assim. N red.	nirb	24.14	0.000	Hemicellulose deg.	xyla	-1.14	0.001

Hemicellulose deg.	mannanase	24.09	0.000	Starch deg.	amyA	-1.15	0.003
Cutin deg.	cutinase	24.07	0.000	Ammonification	urec	-1.16	0.007
Sucrose deg.	invertase_fungi	24.06	0.000	3-hydroxypropionate bicycle	MCM	-1.17	0.004
Starch deg.	amyA	24.01	0.000	Ammonification	urec	-1.17	0.001
Starch deg.	amyA	23.94	0.000	Calvin cycle	FBP_aldolase	-1.18	0.003
Lignin deg.	glx	23.86	0.000	Starch deg.	amyA	-1.18	0.009
Denitrification	nirs	23.85	0.000	Hemicellulose deg.	xyla	-1.19	0.005
Chitin deg.	acetylglucosaminidase	23.8	0.000	Protein deg.	protease_serine_fungi	-1.2	0.000
Hemicellulose deg.	ara	23.68	0.000	Nitrification	hao	-1.22	0.009
Starch deg.	amyA	23.67	0.000	Ammonification	urec	-1.25	0.009
Methanogenesis	mcra	23.66	0.000	Starch deg.	amyA	-1.26	0.009
Calvin cycle	FBPase	23.66	0.000	Hemicellulose deg.	ara	-1.28	0.009
Calvin cycle	GAPDH_Calvin	23.61	0.000	Denitrification	narg	-1.31	0.007
Dissim. N red.	nrfa	23.6	0.000	Starch deg.	cda	-1.31	0.006
Chitin deg.	acetylglucosaminidase	23.59	0.000	Dicarboxylate/4-hydroxybutyrate cycle	fumarase_DiC4HB	-1.31	0.008
Denitrification	nosz	23.59	0.000	Calvin cycle	pgk	-1.34	0.008
Starch deg.	amyA	23.58	0.000	Cellulose deg.	endoglucanase	-1.34	0.009
Denitrification	nirk	23.57	0.000	Starch deg.	amyA	-1.35	0.007
Chitin deg.	acetylglucosaminidase	23.55	0.000	Chitin deg.	acetylglucosaminidase	-1.36	0.010
Starch deg.	amyA	23.55	0.000	Starch deg.	amyA	-1.37	0.007
Starch deg.	amyA	23.54	0.000	Hemicellulose deg.	ara	-1.37	0.009
Bacterial Microcompartments	CsoS1_CcmK	23.53	0.000	Denitrification	narg	-1.37	0.006
Nitro. fix.	nifh	23.49	0.000	Cutin deg.	cutinase	-1.39	0.010
Reductive acetyl CCoA pathway	fthfs	23.48	0.000	Pectin deg.	rgl	-1.39	0.008
Bacterial microcompartments	ccmL	23.43	0.000	Denitrification	narg	-1.41	0.007
Denitrification	nosz	23.41	0.000	Reductive acetyl CCoA pathway	fthfs	-1.43	0.007
Calvin cycle	rubisco	23.29	0.000	Ammonification	urec	-1.44	0.009

Reductive acetyl CCoA pathway	fthfs	11.73	0.000	Cutin deg.	cutinase	-1.45	0.008
Nitro. fix. deg.	nifh	11.62	0.000	Calvin cycle	rubisco	-1.46	0.008
Phospholipid deg.	phospholipase_C_fungi	11.57	0.000	Denitrification	nirs	-1.49	0.009
Assim. N red.	nirb	11.49	0.000	Reductive tricarboxylic acid cycle	AcnA	-1.52	0.008
Denitrification	nosz	11.44	0.000	Calvin cycle	rubisco	-1.62	0.010
Hemicellulose deg.	ara	11.44	0.000	Starch deg.	amyA	-1.74	0.002
Starch deg.	amyA	11.43	0.000	Denitrification	nosz	-4.28	0.001
Chitin deg.	chitin_deacetylase_fungi	11.31	0.000	Bacterial Microcompartments	CsoS1_CcmK	-11.24	0.008
Calvin cycle	tktA	11.19	0.000	Starch deg.	amyA	-11.62	0.006
Calvin cycle	TIM	11.16	0.000	Bacterial Microcompartments	ccmL	-11.89	0.000
Chitin deg.	chitinase	11.12	0.000	Denitrification	norb	-22.14	0.000
Calvin cycle	PRK	11.07	0.010	Chitin deg.	chitinase	-22.19	0.000
Starch deg.	amyA	10.86	0.000	Hemicellulose deg.	ara	-22.85	0.000
Lignin deg.	mnp	10.84	0.000	Starch deg.	amyA	-22.98	0.000
Starch deg.	amyA	1.34	0.006	N Assimilation	nitrate_reductase	-23.03	0.000
Starch deg.	glucoamylase	1.28	0.002	Methanogenesis	mcra	-23.05	0.000
Calvin cycle	FBPase	1.23	0.009	Chitin deg.	acetylglucosaminidase	-23.24	0.000
Starch deg.	glucoamylase	1.2	0.002	Nitro. fix.	nifh	-23.3	0.000
Calvin cycle	rubisco	1.17	0.002	Hemicellulose deg.	xyla	-23.44	0.000
Reductive acetyl CCoA pathway	fthfs	1.14	0.000	Calvin cycle	GAPDH_Calvin	-23.49	0.000
Lignin deg.	phenol_oxidase	1.14	0.007	Chitin deg.	acetylglucosaminidase	-23.52	0.000
Hemicellulose deg.	xylanase	1.13	0.009	Starch deg.	amyA	-23.52	0.000
Dicarboxylate/4-hydroxybutyrate cycle	mdh_DiC4HB	1.12	0.006	Calvin cycle	pgk	-23.53	0.000
Cutin deg.	cutinase	1.12	0.005	Pectin deg.	RgaE	-23.58	0.000
Calvin cycle	PRI	1.11	0.000	Chitin deg.	acetylglucosaminidase	-23.64	0.000
Calvin cycle	FBPase	1.09	0.001	Starch deg.	amyA	-23.66	0.000
Assim. N red.	nira	1.09	0.010	Chitin deg.	chitinase	-23.69	0.000

Denitrification	nosz	1.02	0.010	Pectin deg.	RgaE	-23.71	0.000
Denitrification	narg	1.02	0.009	Nitro. fix.	nifh	-23.71	0.000
Denitrification	nosz	1.01	0.010	Starch deg.	pula	-23.75	0.000
Starch deg.	amyA	1	0.007	Hemicellulose deg.	mannanase	-23.75	0.000
Denitrification	nosz	0.98	0.000	Chitin deg.	acetylglucosaminidase	-23.77	0.000
Reductive tricarboxylic acid cycle	AcnA	0.96	0.003	Starch deg.	amyA	-23.78	0.000
Starch deg.	amyA	0.96	0.004	Assim. N red.	nira	-23.85	0.000
Cutin deg.	cutinase	0.96	0.005	Hemicellulose deg.	xyla	-23.86	0.000
Calvin cycle	rubisco	0.96	0.009	Agar deg.	beta_agarase	-23.88	0.000
Cutin deg.	cutinase	0.95	0.007	Starch deg.	glucoamylase	-23.88	0.000
Calvin cycle	tktA	0.94	0.001	Denitrification	nirs	-23.89	0.000
Denitrification deg.	narg	0.94	0.010	Starch	amyA	-23.89	0.000
Calvin cycle	FBPase	0.94	0.002	Ammonification	gdh	-23.89	0.000
Hemicellulose deg.	xylanase	0.92	0.007	Denitrification	narg	-23.92	0.000
Nitro. fix.	nifh	0.91	0.010	Dicarboxylate/4-hydroxybutyrate cycle	fumarase_DiC4HB	-23.93	0.000
Chitin deg.	chitinase	0.9	0.009	Calvin cycle	rubisco	-23.95	0.000
Vanillin/Lignin deg.	vana	0.89	0.010	Reductive acetyl CCoA pathway	fthfs	-23.97	0.000
Starch deg.	amyA	0.89	0.008	Starch deg.	pula	-24	0.000
Calvin cycle	PRI	0.89	0.010	Starch deg.	amyA	-24.02	0.000
Lignin deg.	phenol_oxidase	0.89	0.000	Starch deg.	amyA	-24.03	0.000
Calvin cycle	GAPDH_Calvin	0.88	0.001	Starch deg.	amyA	-24.03	0.000
Calvin cycle	GAPDH_Calvin	0.88	0.001	Starch deg.	amyA	-24.11	0.000
Calvin cycle	PRK	0.88	0.009	Starch deg.	amyA	-24.13	0.000
Calvin cycle	FBP_aldolase	0.88	0.000	Bacterial Microcompartments	CsoS1_CcmK	-24.14	0.000
Methanogenesis	mcra	0.88	0.006	Reductive acetyl CCoA pathway	fthfs	-24.16	0.000
Chitin deg.	chitinase	0.88	0.007	Hemicellulose deg.	ara	-24.23	0.000
Dissim. N red.	napa	0.88	0.003	Hemicellulose deg.	ara	-24.24	0.000

Starch deg.	cda	0.87	0.006	Cellulose deg.	cellobiase	-24.29	0.000
Calvin cycle	pgk	0.86	0.000	Denitrification	nirk	-24.31	0.000
Calvin cycle	pgk	0.85	0.009	Pectin deg.	exopolygalacturonase_fungi	-24.33	0.000
Pectin deg.	RgaE	0.83	0.001	N Assimilation	nitrate_reductase	-24.34	0.000
Cellulose deg.	endoglucanase	0.82	0.005	Alginate deg.	alginase	-24.34	0.000
Ammonification	urec	0.82	0.002	Starch deg.	cda	-24.38	0.000
Nitro. fix.	nifh	0.82	0.000	Denitrification	nosz	-24.42	0.000
Chitin deg.	endochitinase	0.82	0.004	Nitro. fix.	nifh	-24.44	0.000
Calvin cycle	tktA	0.81	0.003	Lignin deg.	glx	-24.44	0.000
Denitrification	narg	0.81	0.003	Chitin deg.	endochitinase	-24.45	0.000
Denitrification	nosz	0.81	0.003	Starch deg.	amyA	-24.5	0.000
Hemicellulose deg.	xylanase	0.8	0.009	Methanogenesis	mcra	-24.5	0.000
Nitro. fix.	nifh	0.8	0.000	Nitro. fix.	nifh	-24.51	0.000
Chitin deg.	chitinase	0.8	0.000	Phospholipids deg.	phospholipase_D_fungi	-24.52	0.000
Starch deg.	amyA	0.79	0.000	Nitro. fix.	nifh	-24.54	0.000
Denitrification	nosz	0.79	0.000	Pectin deg.	rgl	-24.62	0.000
Hemicellulose deg.	xyla	0.79	0.007	Starch deg.	glucoamylase	-24.64	0.000
Nitro. fix.	nifh	0.78	0.002	Hemicellulose deg.	ara	-24.65	0.000
Cellulose deg.	cellobiase	0.78	0.002	Methanogenesis	mcra	-24.65	0.000
Phospholipid deg.	phospholipase_D_fungi	0.77	0.007	Starch deg.	amyA	-24.68	0.000
Chitin deg.	endochitinase	0.76	0.000	Calvin cycle	rubisco	-24.69	0.000
Starch deg.	amyA	0.76	0.001	Starch deg.	amyA	-24.7	0.000
Denitrification	narg	0.75	0.009	Starch deg.	amyA	-24.71	0.000
Bacterial Microcompartments	CsoS2	0.75	0.002	Dissim. N fix.	nrfa	-24.72	0.000
Nitro. fix.	nifh	0.73	0.006	Starch deg.	amyA	-24.76	0.000
Cellulose deg.	endoglucanase	0.73	0.006	Starch deg.	pula	-24.77	0.000
Starch deg.	amyA	0.73	0.010	Calvin cycle	FBP_aldolase	-24.77	0.000
Assim. N red.	nirb	0.72	0.008	Nitro. fix.	nifh	-24.77	0.000

Nitro. fix.	nifh	0.72	0.005	Bacterial Microcompartments	ccmL	-24.77	0.000
Starch deg.	amyA	0.71	0.007	Starch deg.	amyA	-24.78	0.000
Starch deg.	amyA	0.69	0.005	Cutin deg.	cutinase	-24.79	0.000
Starch deg.	amyA	0.69	0.006	Pectin deg.	endopolygalacturonase_fungi	-24.79	0.000
Calvin cycle	GAPDH_Calvin	0.68	0.006	Hemicellulose deg.	ara	-24.8	0.000
Calvin cycle	TIM	0.68	0.008	Pectin deg.	rgl	-24.8	0.000
Protein deg.	protease_serine_fungi	0.67	0.009	Hemicellulose deg.	mannanase	-24.82	0.000
Lignin deg.	phenol_oxidase	0.64	0.010	Starch deg.	amyA	-24.86	0.000
Methane oxidation	pmoa	0.64	0.004	Chitin deg.	endochitinase	-24.87	0.000
Methanogenesis	mcra	0.63	0.000	Hemicellulose deg.	xylanase	-24.9	0.000
Starch deg.	amyA	0.62	0.008	3-hydroxypropionate bicycle	accD	-24.93	0.000
Starch deg.	amyA	0.6	0.001	Dissim. N fix.	nrfa	-24.93	0.000
Nitro. fix.	nifh	0.6	0.001	Starch deg.	amyA	-24.93	0.000
Calvin cycle	tktA	0.58	0.001	Methanogenesis	mcra	-24.98	0.000
Calvin cycle	tktA	0.36	0.000	Denitrification	nirk	-24.98	0.000
N Assimilation	nitrate_reductase	-0.58	0.002	Chitin deg.	chitinase	-24.98	0.000
Assim. N red.	nirb	-0.6	0.008	Calvin cycle	GAPDH_Calvin	-25.01	0.000
Starch deg.	amyA	-0.6	0.002	Starch deg.	cda	-25.05	0.000
Denitrification	narg	-0.6	0.001	Hemicellulose deg.	mannanase	-25.14	0.000
Denitrification	nosz	-0.61	0.008	Bacterial Microcompartments	CsoS1_CcmK	-25.15	0.000
Denitrification	nirk	-0.61	0.005	Chitin deg.	acetylglucosaminidase	-25.16	0.000
Pectin deg.	pme	-0.62	0.008	Denitrification	narg	-25.16	0.000
Pectin deg.	rgh	-0.62	0.009	Starch deg.	pula	-25.18	0.000
Starch deg.	amyA	-0.64	0.002	Starch deg.	amyA	-25.2	0.000
Denitrification	narg	-0.65	0.000	Calvin cycle	PRI	-25.2	0.000
Starch deg.	amyA	-0.65	0.002	Reductive acetyl CCoA pathway	fthfs	-25.2	0.000

Starch deg.	amyA	-0.68	0.005	Hemicellulose deg.	xylanase	-25.24	0.000
Lignin deg.	ligninase	-0.69	0.000	Nitro. fix.	nifh	-25.27	0.000
Calvin cycle	tktA	-0.69	0.008	Hemicellulose deg.	xylanase	-25.28	0.000
Calvin cycle	FBP_aldolase	-0.72	0.003	Calvin cycle	pgk	-25.3	0.000
Nitro. fix.	nifh	-0.73	0.007	Hemicellulose deg.	ara	-25.32	0.000
Calvin cycle	tktA	-0.76	0.001	Calvin cycle	PRK	-25.32	0.000
Pectin deg.	pme	-0.77	0.009	Nitro. fix.	nifh	-25.36	0.000
Hemicellulose deg.	mannanase	-0.81	0.003	Denitrification	nirk	-25.41	0.000
Starch deg.	amyA	-0.81	0.006	Alginate deg.	alginase	-25.43	0.000
Calvin cycle	tktA	-0.82	0.009	Dissim. N fix.	nrfa	-25.43	0.000
Bacterial Microcompartments	CsoS1_CcmK	-0.82	0.003	Starch deg.	amyA	-25.46	0.000
Ammonification	gdh	-0.83	0.005	Cutin deg.	cutinase	-25.47	0.000
Starch deg.	amyA	-0.83	0.005	Calvin cycle	tktA	-25.48	0.000
Starch deg.	amyA	-0.86	0.005	Starch deg.	amyA	-25.48	0.000
Pectin deg.	RgaE	-0.86	0.001	Starch deg.	amyA	-25.49	0.000
Pectin deg.	pme	-0.87	0.007	Hyaluronic acid deg.	hyaluronidase	-25.5	0.000
Lignin deg.	phenol_oxidase	-0.87	0.001	Chitin deg.	chitinase	-25.51	0.000
Pectin deg.	pectin_lyase_Oomycetes	-0.87	0.006	Starch deg.	amyA	-25.58	0.000
Chitin deg.	acetylglucosaminidase	-0.87	0.001	Pectin deg.	rgl	-25.61	0.000
Cellulose deg.	endoglucanase	-0.88	0.004	Tannins deg.	tannase_Cdeg	-25.61	0.000
Cutin deg.	cutinase	-0.89	0.003	Starch deg.	amyA	-25.64	0.000
Pectin deg.	rgl	-0.89	0.003	Hemicellulose deg.	xylanase	-25.71	0.000
Calvin cycle	FBPase	-0.89	0.003	Nitrification	amoa	-25.76	0.000
Chitin deg.	chitinase	-0.9	0.005	Reductive acetyl CCoA pathway	fthfs	-25.77	0.000
Denitrification	nirk	-0.9	0.003	Hyaluronic acid deg.	hyaluronidase	-25.81	0.000
Starch deg.	amyA	-0.9	0.004	Denitrification	narg	-25.89	0.000
Ammonification	gdh	-0.9	0.009	Starch deg.	amyA	-25.95	0.000
Hemicellulose deg.	ara	-0.9	0.007	Starch deg.	amyA	-26.1	0.000

Dissim. N fix.	napa	-0.9	0.010	Protein deg.	protease_serine_fungi	-26.26	0.000
Starch deg.	amyA	-0.91	0.001	Calvin cycle	FBP_aldolase	-26.32	0.000
Starch deg.	amyA	-0.91	0.001	Calvin cycle	PRI	-26.4	0.000
Calvin cycle	tktA	-0.91	0.001	Reductive acetyl CCoA pathway	codh	-26.4	0.000
Calvin cycle	tktA	-0.91	0.000	Reductive acetyl CCoA pathway	fthfs	-26.56	0.000
Pectin deg.	pme	-0.91	0.001	Starch deg.	pula	-26.57	0.000
Starch deg.	amyA	-0.92	0.001	Hemicellulose deg.	xylanase	-26.68	0.000
Dissim. N fix.	napa	-0.92	0.003	Assim. N red.	nasa	-26.71	0.000
Hemicellulose deg.	xylanase	-0.92	0.006	Hemicellulose deg.	ara	-26.85	0.000
Lignin deg.	ligninase	-0.92	0.005	Cellulose deg.	cellobiase	-27.15	0.000
Starch deg.	amyA	-0.92	0.000	Dissim. N fix.	nrfa	-27.21	0.000
Calvin cycle	TIM	-0.92	0.003	Agar deg.	beta_agarase	-27.25	0.000
Vanillin/Lignin deg.	vana	-0.93	0.001	Lignin deg.	phenol_oxidase	-27.49	0.000
Cellulose deg.	cellobiase	-0.93	0.000	Cellulose deg.	exoglucanase	-27.67	0.000
Cutin deg.	cutinase	-0.93	0.000	Hemicellulose deg.	xylanase	-27.67	0.000
Terpenes deg.	limeh	-0.93	0.007	Ammonification	gdh	-27.68	0.000
Pectin deg.	pec_Cdeg	-0.94	0.006	Reductive tricarboxylic acid cycle	frdA_rTCA	-28.35	0.000

Appendix C

Table C.1. The GPS locations of asymptomatic kauri trees selected for soil sampling for the kauri seedling bioassays.

Kauri tree ID	Latitude	Longitude	*P. agathidicida* detection
A1	-35.60027778	173.5280556	Positive
A2	-35.66222222	173.5602778	Negative
A3	-35.66194444	173.5602778	Negative
A4	-35.66194444	173.5605556	Positive
A5	-35.66222222	173.5605556	Positive
A6	-35.6625000	173.5608333	Positive
A7	-35.60083333	173.5266667	Positive
A8	-35.60111111	173.5266667	Positive
A9	-35.60111111	173.5266667	Positive
A10	-35.60111111	173.5266667	Positive
A11	-35.60111111	173.5266667	Negative
A12	-35.67555556	173.5605556	Positive
A13	-35.67555556	173.5605556	Negative
A14	-35.67472222	173.5611111	Negative
A15	-35.67555556	173.5608333	Negative
A16	-35.67583333	173.5608333	Negative
A17	-35.67527778	173.5611111	Negative
A18	-35.67527778	173.5613889	Negative

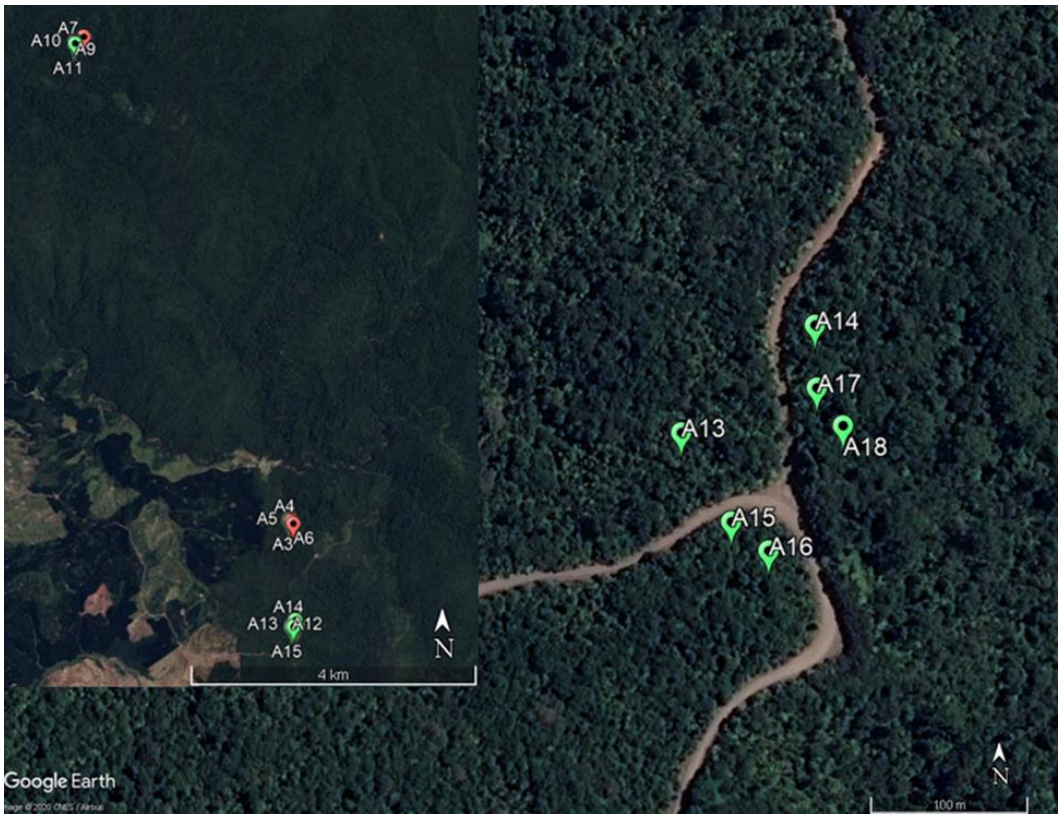

Figure C.1. The locations of kauri across Waipoua Forest (Northland Region, New Zealand) which were selected for soil sampling. The smaller image insert shows the locations of all the 18 kauri trees which were originally targeted for soil collection. The six sites shown in the larger main image are kauri whose soil samples tested negative for *P. agathidicida* presence and were used as potting mix in the seedling bioassays.

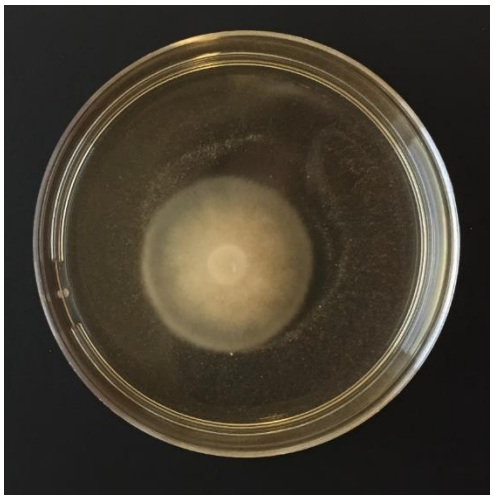

Figure C.2. A mycelial mat of *P. agathidicida* grown in 2% concentrate V8 broth which was used as an inoculum source to infect kauri seedlings for the seedling bioassay.

Table C.2. The results of real-time PCR and soil baiting bioassays used detect the presence of *P. agathidicida* in each soil sample. The cycling threshold values and *P. agathidicida* DNA quantity for each DNA sample assayed are as shown as mean ± standard error.

Kauri tree ID	Ct	Quantity (fentogram)	R2	Efficiency (%)	Soil baiting
A1	32.05 ± 1.13	1061.68 ± 560.79	0.99	96.24	Negative
A2	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A3	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A4	32.86 ± 2.12	893.65 ± 762.29	0.99	96.24	Negative
A5	32.76 ± 0.23	699.02 ± 74.30	0.99	96.24	Negative
A6	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A7	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Positive
A8	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Positive
A9	31.51 ± 0.38	11.39 ± 2.76	0.99	96.24	Positive
A10	33.36 ± 0.06	3.20 ± 0.12	0.99	96.24	Positive
A11	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A12	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Positive
A13	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A14	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A15	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A16	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A17	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative
A18	0.00 ± 0.00	0.00 ± 0.00	0.99	96.24	Negative

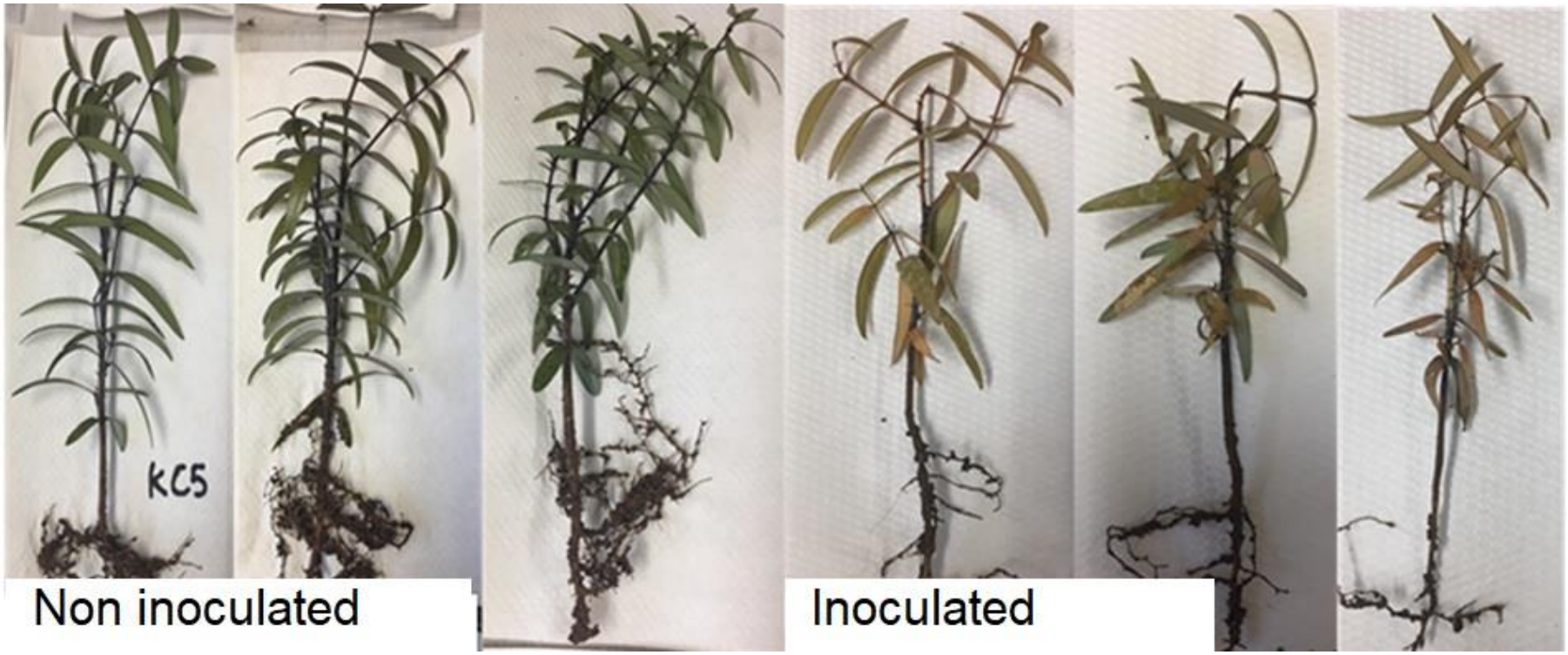

Figure C.3. Example images of control and inoculated kauri seedlings 6 weeks post inoculation with *P. agathidicida.*

Table C.3. The results of real-time PCR assays used to quantify the abundance of *P. agathidicida* cDNA in soils from inoculated seedlings six weeks post infection. Results for the quantity of *P. agathidicida* cDNA detected are expressed as femtograms, calculated by multiplying their nanogram values by 1e+6. All control seedlings had an undetermined Ct value and a cDNA value of 0.00fg, testing negative for the presence of *P. agathidicida*. Therefore their results are not shown in the table.

Seedling ID	Ct mean ± SD	Quantity mean ± SD (fg)	Efficiency %	R2
i1	33.62 ± 0.07	29.23 ± 1.06	89	0.99
i2	32.44 ± 0.23	56.43 ± 6.66	89	0.99
i3	35.45 ± 0.45	6.90 ± 3.98	89	0.99
i4	32.88 ± 0.21	44.33 ± 4.88	89	0.99
i5	37.75 ± 0.37	3.19 ± 0.59	89	0.99
i6	32.29 ± 0.27	61.71 ± 9.33	89	0.99
i7	36.68 ± 0.29	5.67 ± 0.89	89	0.99
i8	34.10 ± 0.63	24.94 ± 6.86	89	0.99
i9	31.81 ± 0.14	78.78 ± 6.17	89	0.99
i10	34.37 ± 0.10	19.47 ± 1.08	89	0.99
i11	32.48 ± 0.09	54.44 ± 2.67	89	0.99
i12	33.48 ± 0.21	31.91 ± 3.54	89	0.99
i13	33.17 ± 0.24	38.00 ± 4.77	89	0.99
i14	36.93 ± 1.21	6.46 ± 3.52	89	0.99
i15	36.73 ± 0.19	5.41 ± 0.56	89	0.99
i17	36.00 ± 0.14	6.13 ± 2.31	89	0.99
i18	32.10 ± 0.35	69.48 ± 14.07	89	0.99
i19	31.98 ± 0.23	72.32 ± 8.94	89	0.99
i20	33.74 ± 0.20	27.72 ± 3.01	89	0.99
i22	34.06 ± 0.15	23.10 ± 1.87	89	0.99
i23	31.77 ± 0.42	84.55 ± 19.77	89	0.99
i24	34.70 ± 0.29	29.63 ± 4.25	89	0.99
i25	32.93 ± 0.46	78.10 ± 20.37	89	0.99
i26	36.52 ± 0.18	8.30 ± 2.31	89	0.99
i27	36.10 ± 0.60	9.04 ± 2.76	89	0.99
i29	34.49 ± 0.36	33.45 ± 5.69	89	0.99
i30	34.96 ± 0.31	25.87 ± 4.13	89	0.99

Table C.4. The results of DESeq2 analysis showing fungal OTUs with significantly different abundances between soils from inoculated and non-inoculated seedlings. OTUs with a negative mean difference show taxa significantly higher in inoculated seedlings, those with positive values are significantly lower in inoculated seedlings.

Taxa ID of fungal OTU	Mean difference	P value
Mortierellomycota	0.0401	0.001
Mortierellomycetes	0.0401	0.001
Saccharomycetes	-0.0028	0.006
Dothideomycetes	-0.0034	0.004
Mucoromycetes	0.0005	0.000
Rozellomycotina_cls_Incertae_sedis	0.0010	0.001
Mortierellales	0.0401	0.001
Saccharomycetales	-0.0028	0.006
Botryosphaeriales	-0.0042	0.000
Mucorales	0.0005	0.000
Capnodiales	0.0002	0.024
Thelebolales	0.0004	0.047
Filobasidiales	-0.0004	0.002
Xylariales	-0.0007	0.001
Mortierellaceae	0.0401	0.001
Mucoraceae	0.0003	0.000
Pyronemataceae	0.0004	0.047
Hyaloscyphaceae	-0.0033	0.001
Pyronemataceae	0.0006	0.024
Saccharomycetales_fam_Incertae_sedis	-0.0004	0.000
Strophariaceae	0.0000	0.031
Thermoascaceae	-0.0006	0.008
Piskurozymaceae	-0.0004	0.002
Mortierella	0.0401	0.001
Mucor	0.0003	0.000
Candida	-0.0004	0.000
Byssochlamys	-0.0006	0.008

Table C.5. The results of DESeq2 analysis showing bacterial OTUs with significantly different abundances between soils from inoculated and non-inoculated seedlings. OTUs with a negative mean difference show taxa significantly higher in inoculated seedlings, those with positive values are significantly lower in inoculated seedlings.

Taxa ID of bacterial OTU	Mean difference	P value	Taxa ID of bacterial OTU	Mean difference	P value
Ammoniphilus	-0.0003	0.001	Rhodocyclaceae	-0.0022	0.000
Aquitalea	-0.0062	0.000	Rhodocyclales	-0.0022	0.000
Azospirillum	-0.0006	0.000	Rickettsiales	-0.0067	0.000
Bacillaceae	-0.0069	0.000	Rubrivivax	-0.0002	0.023
Bacillales	-0.01	0.000	Ruminococcaceae	-0.0008	0.000
Bacilli	-0.0101	0.000	Sinomonas	-0.0011	0.018
Bacillus	-0.0069	0.000	Streptacidiphilus	-0.0002	0.018
Bacteroidales	-0.0018	0.000	Veillonellaceae	-0.0007	0.000
Bacteroidia	-0.0018	0.000	Xanthomonadaceae	-0.0023	0.002
Betaproteobacteria	-0.0341	0.000	Acetobacteraceae	0.0016	0.021
Bifidobacteriaceae	-0.0003	0.012	Acidimicrobiales	0.0011	0.000
Bifidobacteriales	-0.0003	0.012	Actinoallomurus	0.0002	0.009
Bifidobacterium	-0.0003	0.012	Actinoallomurus	0.0002	0.009
Brevibacteriaceae	-0.0004	0.018	Actinobacteria	0.0286	0.000
Brevibacterium	-0.0004	0.018	Actinomadura	0.0002	0.019
Burkholderiales	-0.0255	0.000	Actinomycetales	0.0063	0.032
Caloramator	-0.0012	0.000	Actinosynnemataceae	0.0002	0.005
Cellulomonadaceae	-0.0005	0.000	Alphaproteobacteria	0.0004	0.002
Cellulomonas	-0.0005	0.000	Beijerinckiaceae	0.0005	0.004
Bacillus cereus	-0.0002	0.008	Bradyrhizobiaceae	0.0013	0.000
Clostridia	-0.0186	0.000	Bradyrhizobium	0.0010	0.001
Clostridiaceae	-0.0144	0.000	Burkholderia bryophila	0.0019	0.011
Clostridiales	-0.0186	0.000	Candidatus Solibacter	0.0040	0.000
Clostridium	-0.0132	0.000	Candidatus	0.0010	0.037
Bacillus coagulans	-0.0025	0.032	Chloracidobacteria	0.0002	0.020
Comamonadaceae	-0.0092	0.000	Chloroflexi	0.0002	0.006
Cupriavidus	-0.0041	0.000	Chthoniobacteraceae	0.0064	0.017
Dechloromonas	-0.0002	0.001	Chthoniobacterales	0.0075	0.011
Deltaproteobacteria	-0.0069	0.000	Conexibacteraceae	0.0021	0.000
Denitrobacter	-0.0001	0.002	Dactylosporangium	0.0002	0.001
Desulfobacterales	-0.0002	0.010	Dokdonella	0.0006	0.002
Desulfobulbaceae	-0.0002	0.010	Edaphobacter	0.0009	0.000
Desulfobulbus	-0.0002	0.010	Frankia	0.0001	0.001
Desulfosporosinus	-0.0015	0.000	Frankiaceae	0.0010	0.000
Desulfuromonadales	-0.0086	0.000	Gaiellaceae	0.0046	0.001
Enterobacteriaceae	-0.0033	0.000	Gaiellales	0.0003	0.001
Enterobacteriales	-0.0033	0.000	Gemmatimonadales	0.0003	0.005

Firmicutes	-0.0287	0.000	Hyphomicrobiaceae	0.0011	0.008
Bacillus flexus	-0.0004	0.034	Kribbella	0.0002	0.002
Gallionella	-0.0003	0.000	Ktedonobacteraceae	0.0009	0.003
Gallionellaceae	-0.0003	0.000	Ktedonobacteria	0.0012	0.022
Gallionellales	-0.0003	0.000	Labrys	0.0002	0.017
Gammaproteobacteria	-0.0002	0.000	Lentzea	0.0002	0.008
gelatinosus	-0.0002	0.023	Micromonosporaceae	0.0006	0.000
Geobacter	-0.0086	0.000	Mycobacterium	0.0023	0.000
Geobacteraceae	-0.0086	0.000	Mycobacterium celatum	0.0005	0.012
Geothrix	-0.0003	0.000	Nocardia	0.0002	0.001
Herbaspirillum	-0.0012	0.000	Nocardiaceae	0.0002	0.001
Holophagaceae	-0.0018	0.000	Patulibacteraceae	0.0005	0.000
Holophagae	-0.0018	0.000	Pedomicrobium	0.0005	0.020
Holophagales	-0.0018	0.000	Phyllobacteriaceae	0.0004	0.012
Janthinobacterium	-0.0039	0.000	Phyllobacterium	0.0001	0.014
Limnohabitans	-0.0002	0.000	Pilimelia	0.0002	0.008
Magnetospirillum	-0.0007	0.000	Pleomorphomonas	0.0004	0.000
meridiei	-0.0015	0.000	Pseudomonas	0.0003	0.026
Micrococcaceae	-0.0012	0.018	Pseudonocardia	0.0004	0.000
Neisseriaceae	-0.0063	0.000	Pseudonocardiaceae	0.0004	0.000
Neisseriales	-0.0063	0.000	Rhizobiaceae	0.0004	0.050
nitroreducens	-0.0022	0.000	Rhizobiales	0.0252	0.000
Nitrosomonadaceae	-0.0002	0.000	Rhodoplanes	0.0105	0.004
Nitrospira	-0.0002	0.046	Rhodospirillaceae	0.0153	0.000
Nitrospiraceae	-0.0002	0.001	Rhodospirillales	0.0170	0.000
Nitrospirae	-0.0002	0.046	Solibacteraceae	0.0005	0.002
Nitrospirales	-0.0002	0.046	Solibacterales	0.0045	0.000
Oxalobacteraceae	-0.015	0.000	Solibacteres	0.0045	0.000
Paenibacillaceae	-0.0017	0.000	Solirubrobacter	0.0003	0.021
Paenibacillus	-0.0014	0.000	Solirubrobacteraceae	0.0003	0.014
Paludibacter	-0.0016	0.000	Solirubrobacterales	0.0041	0.000
Pelosinus	-0.0004	0.000	Sphingomonadaceae	0.0010	0.002
Peptococcaceae	-0.0015	0.000	Sphingomonadales	0.0012	0.002
Peptostreptococcaceae	-0.0003	0.002	Sphingomonas	0.0001	0.018
Planococcaceae	-0.0011	0.009	Streptomyces	0.0006	0.005
Planococcus	-0.001	0.016	Thermoleophilia	0.0121	0.000
Porphyromonadaceae	-0.0016	0.000	Thermomonosporaceae	0.0004	0.006
Propionivibrio	-0.0005	0.000	Verrucomicrobia	0.0078	0.014
Rhodanobacter	-0.0004	0.000	Xanthobacteraceae	0.0003	0.010

Appendix D

Supplementary Methods D.1. Bacterial and fungal selective agar recipes.

Bacterial selective agar, per litre

- 28 g nutrient agar (Oxoid Ltd., UK)
- 200 mg of pentachloronitrobenzene (PCNB) (Sigma Aldrich, Missouri USA)
- 400 µl of pimaricin (Sigma Aldrich, Missouri USA)
- 50 mg of hymexazol (Tokyo Chemical Industry Co., Ltd, Japan)

Fungal selective agar, per litre

- 39 g of potato dextrose agar (Oxoid Ltd., UK)
- 250 mg of chloramphenicol (Sigma Aldrich, Missouri USA)
- 90 mg of streptomycin sulphate (Sigma Aldrich, Missouri USA)
- 50 mg of hymexazol (Tokyo Chemical Industry Co., Ltd, Japan)

Figure D.1. The fungal strains found to significantly inhibit *P. agathidicida* growth in dual culture bioassays. Photographed on PDA media after 2 weeks of growth at 22°C in darkness.

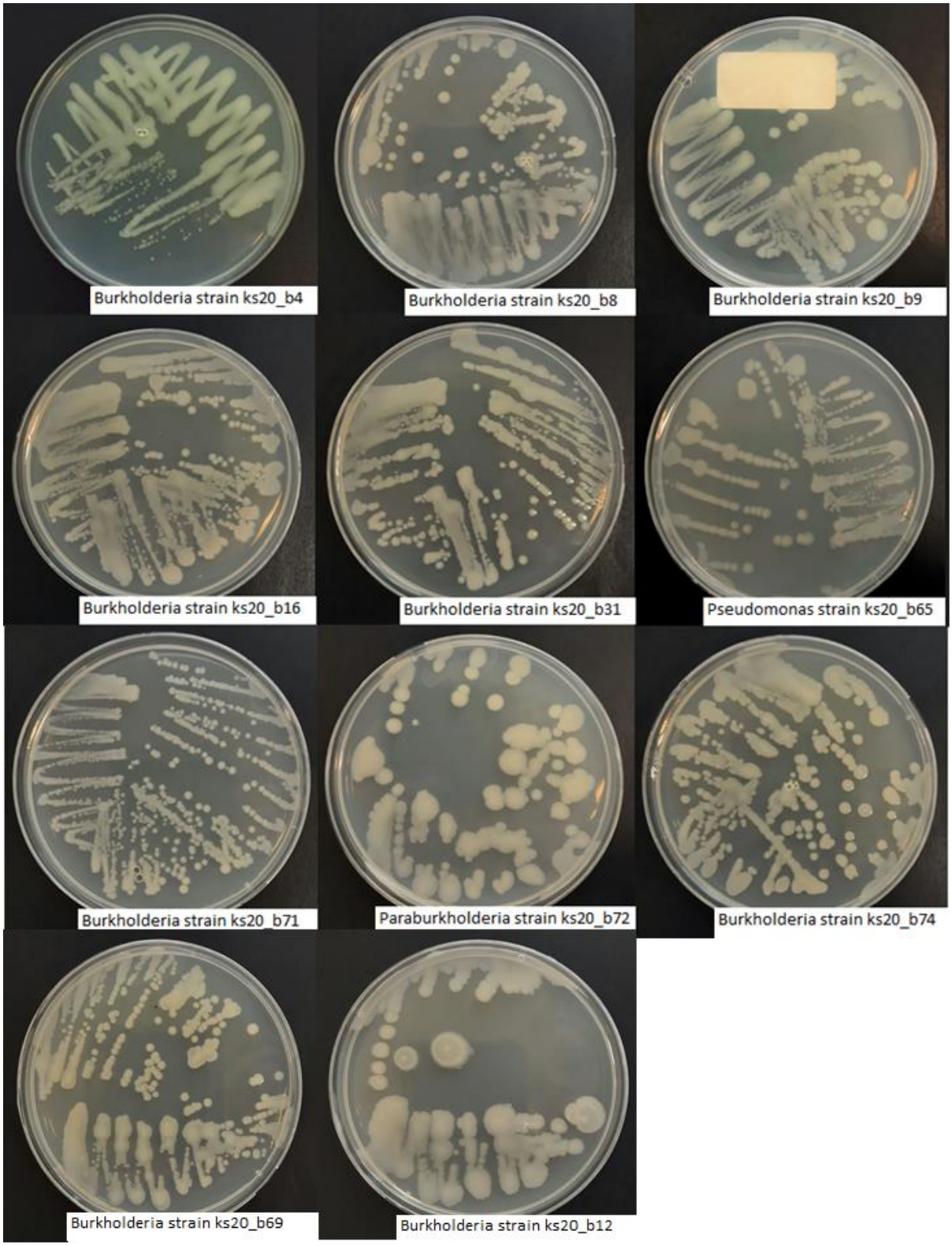

Figure D.2. The bacterial strains found to significantly inhibit *P. agathidicida* growth in dual culture bioassays. Photographed on NA media after 72 hours of growth at 22°C in darkness.

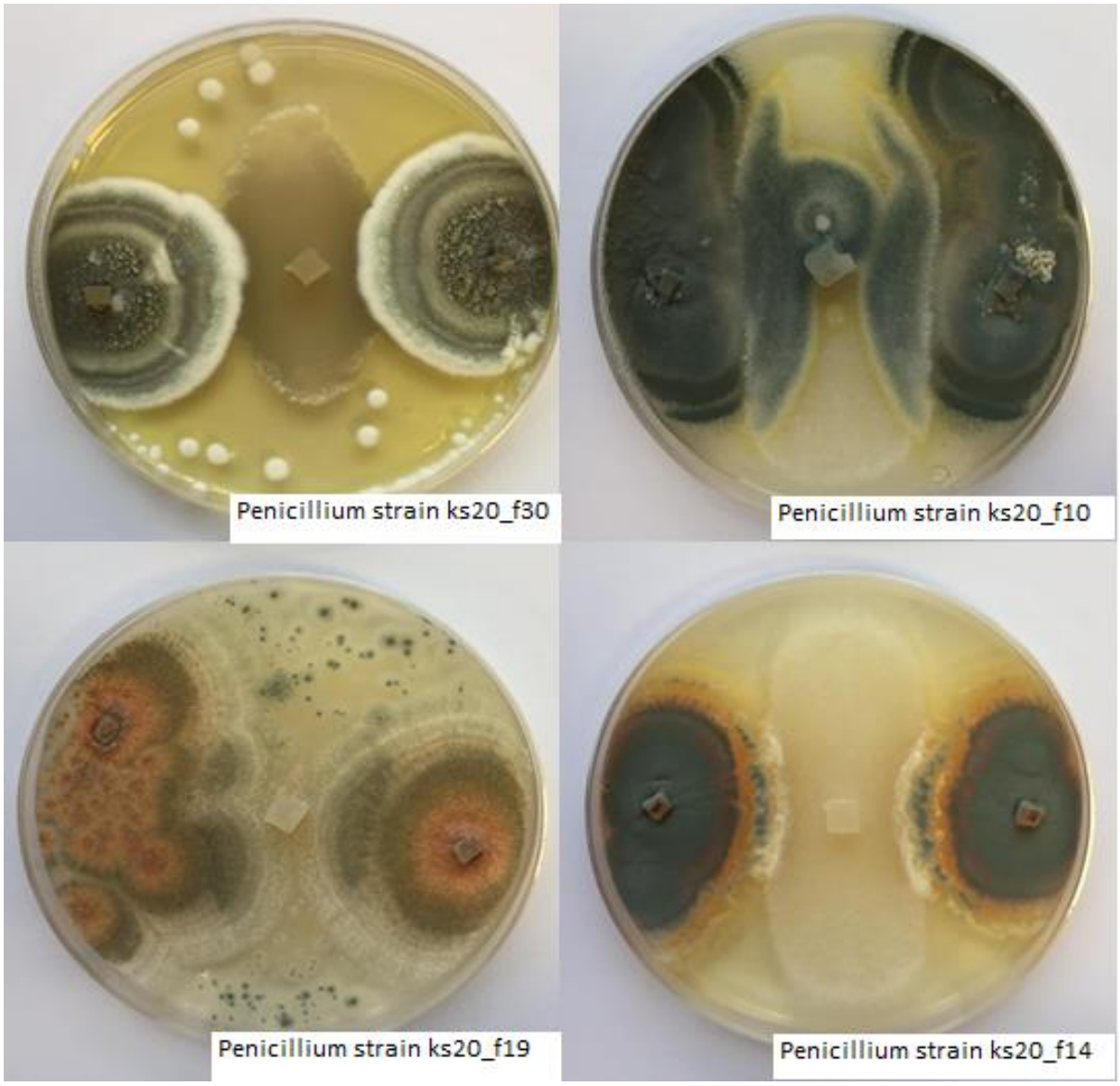

Figure D.3. Images of dual culture bioassays showing fungal strains which grew over *P. agathidicida* mycelium.

Table D.1. The mean ± standard error mycelial inhibition values (%) of *P. agathidicida* cultures when grown in dual culture, culture filtrate and split plate bioassays with each of the 9 fungal strains under study. Significance tested using 2 sample t-tests.

	Dual culture bioassay		Culture filtrate bioassay		Split plate bioassay	
Fungal strain ID	**Mycelial inhibition %**	**Significance**	**Mycelial inhibition %**	**Significance**	**Mycelial inhibition %**	**Significance**
Penicillium strain ks20_f18	58.28 ± 1.55	t-value = 33.80, p-value = 0.000	4.05 ± 2.88	t-value = 1.02, p-value = 0.329	14.41 ± 2.42	t-value= 5.55, p-value = 0.012
Penicillium strain ks20_f15	48.03 ± 1.71	t-value = 25.74, p-value = 0.000	6.23 ± 3.94	t-value = 1.30, p-value = 0.241	20.26 ± 2.64	t-value= 7.22, p-value = 0.000
Penicillium strain ks20_f14	54.63 ± 0.80	t-value = 49.48, p-value = 0.000	7.03 ± 2.80	t-value = 1.80, p-value = 0.088	19.53 ± 4.83	t-value= 7.22, p-value = 0.000
Penicillium strain ks20_f19	52.24 ± 1.47	t-value = 31.57, p-value = 0.000	5.44 ± 2.82	t-value = 1.39, p-value = 0.182	19.09 ± 4.60	t-value= 4.06, p-value = 0.005
Penicillium strain ks20_f52	55.27 ± 1.26	t-value = 37.60, p-value = 0.000	0.57 ± 2.65	t-value = 0.15, p-value = 0.882	20.33 ± 4.76	t-value= 4.19, p-value = 0.006
Penicillium strain ks20_f54	51.29 ± 1.40	t-value = 32.25, p-value = 0.000	5.95 ± 2.48	t-value = 1.62, p-value = 0.124	16.91 ± 6.66	t-value= 2.51, p-value = 0.054
Penicillium strain ks20_f10	50.18 ± 1.74	t-value = 26.50, p-value = 0.000	6.4 ± 2.44	t-value = 1.75, p-value = 0.098	18.68 ± 4.79	t-value= 3.82, p-value = 0.009
Penicillium strain ks20_f30	57.27 ± 1.20	t-value = 40.46, p-value = 0.000	5.82 ± 2.52	t-value = 1.57, p-value = 0.134	18.94 ± 3.82	t-value= 4.81, p-value = 0.003
Penicillium strain ks20_f20	52.47 ± 1.49	t-value = 31.42, p-value = 0.000	33.72 ± 4.95	t-value = 5.98, p-value = 0.000	19.90 ± 6.92	t-value= 2.85, p-value = 0.046

Table D.2. The mean ± standard error mycelial inhibition values (%) of P. *agathidicida* cultures when grown in dual culture, culture filtrate and split plate bioassays with each of the 11 bacterial strains under study. Significance tested using 2 sample t-tests.

	Dual culture bioassays		Culture filtrate bioassays		Split plate bioassays	
Bacterial strain ID	**Mycelial inhibition %**	**Significance**	**Mycelial inhibition %**	**Significance**	**Mycelial inhibition %**	**Significance**
Burkholderia strain ks20_b16	57.87 ± 1.26	t-value = 29.61, p-value = 0.000	11.63 ± 3.47	t-value = 2.98, p-value = 0.009	14.77 ± 3.85	t-value= 3.72, p-value = 0.006
Burkholderia strain ks20_b12	59.91 ± 1.84	t-value = 25.27, p-value = 0.000	10.44 ± 3.96	t-value = 2.40, p-value = 0.031	14.48 ± 3.44	t-value= 4.06, p-value = 0.002
Burkholderia strain ks20_b4	56.66 ± 2.10	t-value = 22.01, p-value = 0.000	13.58 ± 2.92	t-value = 3.96, p-value = 0.001	19.87 ± 4.49	t-value= 4.33, p-value = 0.003
Burkholderia strain ks20_b8	55.75 ± 1.81	t-value = 23.74, p-value = 0.000	14.40 ± 2.50	t-value = 4.67, p-value = 0.000	18.75 ± 4.30	t-value= 4.26, p-value = 0.002
Burkholderia strain ks20_b9	58.13 ± 1.88	t-value = 24.20, p-value = 0.000	16.43 ± 1.71	t-value = 6.62, p-value = 0.000	21.03 ± 3.17	t-value= 6.35, p-value = 0.000
Burkholderia strain ks20_b69	59.78 ± 1.37	t-value = 29.57, p-value = 0.000	13.69 ± 3.19	t-value = 3.74, p-value = 0.002	16.39 ± 2.73	t-value= 5.69, p-value = 0.000
Pseudomonas strain ks20_b65	51.88 ± 1.16	t-value = 27.49, p-value = 0.000	13.01 ± 3.31	t-value = 3.46, p-value = 0.003	9.01 ± 6.52	t-value= 1.38, p-value = 0.201
Burkholderia strain ks20_b31	53.19 ± 1.77	t-value = 22.96, p-value = 0.000	13.4 ± 3.74	t-value = 3.23, p-value = 0.006	15.62 ± 5.31	t-value= 2.89, p-value = 0.018
Burkholderia strain ks20_b71	60.88 ± 3.22	t-value = 17.15, p-value = 0.000	9.32 ± 2.52	t-value = 3.00, p-value = 0.009	12.25 ± 3.02	t-value= 3.87, p-value = 0.003
Burkholderia strain ks20_b74	57.31 ± 1.73	t-value = 25.10, p-value = 0.000	10.67 ± 2.88	t-value = 3.14, p-value = 0.005	16.44 ± 2.66	t-value= 5.81, p-value = 0.000
Paraburkholderia strain ks20_b72	51.52 ± 1.25	t-value = 26.47, p-value = 0.000	6.71 ± 2.70	t-value = 2.07, p-value = 0.059	11.51 ± 3.76	t-value= 2.97, p-value = 0.014

Table D.3. The volatile organic compounds that were identified to be produced by the microbial strains tested using HS-SPME/GC MS analysis. Only compounds which had a > 85% match rate and had a linear retention index (LRI) within 5% of their reported literature values are shown.

Strain	Name	% peak area	Measured LRI	Literature LRI	Hit %	Class
Burkholderia strain ks20_b4	1-Undecene (CAS num: 821-95-4)	0.56 ± 0.04	1091	1087	98	Alkene
	Dimethyl trisulfide (CAS num: 3658-80-8)	0.19 ± 0.01	972	971	94	Organic trisulfide
	2-butan-2-yl-3-methoxy-5-(2-methylpropyl) pyrazine (CAS num: 870543-98-9)	0.67 ± 0.11	1448	1463	90	Pyrazine
	S-Methyl propanethioate (CAS num: 5925-75-7)	0.26 ± 0.02	800	785	86	Thiocarboxylic acid
Burkholderia strain ks20_b8	1-Undecene (CAS num: 821-95-4)	0.25 ± 0.01	1091	1087	97	Alkene
	2-Pentadecanone (CAS num: 2345-28-0)	0.14 ± 0.00	1702	1698	96	Ketone
	beta-Myrcene (CAS num: 123-35-3)	0.12 ± 0.01	993	981	95	Monoterpenoid
Burkholderia strain ks20_b9	1-Undecene (CAS num: 821-95-4)	0.60 ± 0.04	1091	1087	97	Alkene
	2-butan-2-yl-3-methoxy-5-(2-methylpropyl)pyrazine (CAS num: 870543-98-9)	0.18 ± 0.00	1449	1463	88	Pyrazine
	Dimethyl trisulfide (CAS num: 3658-80-8)	0.19 ± 0.03	972	971	93	Organic trisulfide
	S-Methyl propanethioate (CAS num: 5925-75-7)	0.25 ± 0.02	801	785	85	Thiocarboxylic acid
Burkholderia strain ks20_b12	beta-Myrcene (CAS num: 123-35-3)	0.13 ± 0.00	992	981	96	Monoterpenoid
	beta Ocimene (CAS num: 3779-61-1)	0.07 ± 0.00	1050	1050	98	Monoterpenoid
	alpha Ocimene (CAS num: 6874-10-8)	0.03 ± 0.00	1040	1050	97	Monoterpenoid
	1-Undecene (CAS num: 821-95-4)	0.52 ± 0.01	1091	1087	98	Alkene
	2-Pentadecanone (CAS num: 2345-28-0)	0.09 ± 0.01	1701	1698	92	Ketone
Burkholderia strain ks20_b71	beta-Myrcene (CAS num: 123-35-3)	0.06 ± 0.01	991	981	96	Monoterpenoid

	beta Ocimene (CAS num: 3779-61-1)	0.04 ± 0.00	1049	1050	98	Monoterpenoid
	1-Undecene (CAS num: 821-95-4)	0.30 ± 0.04	1089	1087	98	Alkene
	2-butan-2-yl-3-methoxy-5-(2-methylpropyl) pyrazine (CAS num: 870543-98-9)	3.48 ± 0.34	1448	1463	91	Pyrazine
	7-Oxabicyclo[4.1.0]heptane, 1-(2,3-dimethyl-1,3-butadienyl)-2,2,6-trimethyl-, (E)- (CAS num: 59744-12-6)	1.08 ± 0.04	1428	1461	84	Sesquiterpenoid
	2-Decyloxirane (CAS num: 2855-19-8)	0.14 ± 0.03	1313	1307	95	Epoxide
Burkholderia strain ks20_b74	beta-Myrcene (CAS num: 123-35-3)	0.10 ± 0.00	991	981	96	Monoterpenoid
	beta Ocimene (CAS num: 3779-61-1)	0.07 ± 0.00	1049	1050	98	Monoterpenoid
	alpha Ocimene (CAS num: 6874-10-8)	0.03 ± 0.00	1039	1050	98	Monoterpenoid
	Pentamethylcyclopentadiene (CAS num: 4045-44-7)	0.05 ± 0.00	1018	983	89	Alkene
	1-Undecene (CAS num: 821-95-4)	0.28 ± 0.03	1089	1087	98	Alkene
	3-Chloropropionic acid, 4-isopropylphenyl ester	0.18 ± 0.01	1471	1507	86	Benzenoid
	2-butan-2-yl-3-methoxy-5-(2-methylpropyl)pyrazine (CAS num: 870543-98-9)	3.47 ± 0.01	1448	1463	92	Pyrazine
	S-Methyl propanethioate (CAS num: 5925-75-7)	0.03 ± 0.01	801	785	87	Thiocarboxylic acid
Burkholderia strain ks20_b16	beta-Myrcene (CAS num: 123-35-3)	0.12 ± 0.01	992	981	95	Monoterpenoid
	beta Ocimene (CAS num: 3779-61-1)	0.08 ± 0.00	1050	1050	97	Monoterpenoid
	alpha Ocimene (CAS num: 6874-10-8)	0.04 ± 0.00	1040	1052	98	Monoterpenoid
	Pentamethylcyclopentadiene (CAS num: 4045-44-7)	0.05 ± 0.00	1019	983	92	Alkene
	1-Undecene (CAS num: 821-95-4)	0.18 ± 0.01	1091	1087	97	Alkene
	2-Pentadecanone (CAS num: 2345-28-0)	0.03 ± 0.00	1702	1698	94	Ketone
	3-Chloropropionic acid, 4-isopropylphenyl ester	0.23 ± 0.01	1472	1507	88	Benzenoid

	D-Limonene (CAS num: 5989-27-5)	0.02 ± 0.00	1030	1031	96	Monoterpenoid
	S-Methyl propanethioate (CAS num: 5925-75-7)	0.005 ± 0.00	801	785	87	Thiocarboxylic acid
Burkholderia strain ks20_b31	beta-Myrcene (CAS num: 123-35-3)	0.12 ± 0.00	992	981	96	Monoterpenoid
	beta Ocimene (CAS num: 3779-61-1)	0.07 ± 0.01	1050	1050	98	Monoterpenoid
	alpha Ocimene (CAS num: 6874-10-8)	0.04 ± 0.00	1040	1052	98	Monoterpenoid
	Pentamethylcyclopentadiene (CAS num: 4045-44-7)	0.05 ± 0.00	1019	983	93	Alkene
	1-Undecene (CAS num: 821-95-4)	0.32 ± 0.02	1091	1087	98	Alkene
	3-Chloropropionic acid, 4-isopropylphenyl ester	0.24 ± 0.00	1474	1544	99	Benzenoid
	2-butan-2-yl-3-methoxy-5-(2-methylpropyl)pyrazine (CAS num: 870543-98-9)	3.33 ± 0.07	1449	1463	92	Pyrazine
	S-Methyl propanethioate (CAS num: 5925-75-7)	0.05 ± 0.01	800	785	86	Thiocarboxylic acid
Pseudomonas strain ks20_b65	Octa-2,4,6-triene (CAS num: 15192-80-0)	0.37 ± 0.02	935	925	97	Terpene
	beta-Myrcene (CAS num: 123-35-3)	0.05 ± 0.03	993	981	92	Monoterpenoid
	1,3,5-Heptatriene (CAS num: 17679-93-5)	1.72 ± 0.01	792	781	97	Alkene
	2-Heptanone (CAS num: 110-43-0)	0.25 ± 0.01	893	889	97	Ketone
	2-Nonanone (CAS num: 821-55-6)	0.08 ± 0.00	1094	1091	94	Ketone
	2-Methylbutanoic acid (CAS num: 116-53-0)	0.22 ± 0.10	866	894	95	Fatty acyl
	Dimethyl trisulfide (CAS num: 3658-80-8)	1.38 ± 0.03	972	971	94	Organic trisulfide
	Ethyl methyl disulfide (CAS num: 20333-39-5)	0.08 ± 0.00	837	833	91	Organic disulfide
	1-Methoxyundecane (CAS num: 7289-53-4)	0.14 ± 0.01	1281	1290	90	Organic oxide
	2,4-Di-tert-butylphenol (CAS num: 96-76-4)	0.03 ± 0.00	1518	1513	92	Benzenoid

	2-Ethyl-5-methylpyrazine (CAS num: 13360-64-0)	0.37 ± 0.03	1004	1002	95	Pyrazine
	Dimethyltetrasulfane (CAS num: 5756-24-1)	0.08 ± 0.03	1222	1200	91	Organic trisulfide
Burkholderia strain ks20_b69	2-butan-2-yl-3-methoxy-5-(2-methylpropyl) pyrazine (CAS num: 870543-98-9)	2.22 ± 0.03	1451	1463	91	Pyrazine
	beta-Myrcene (CAS num: 123-35-3)	0.08 ± 0.00	993	981	96	Monoterpenoid
	Dimethyl trisulfide (CAS num: 3658-80-8)	0.04 ± 0.00	973	971	93	Organic trisulfide
	beta Ocimene (CAS num: 3779-61-1)	0.05 ± 0.01	1051	1050	97	Monoterpenoid
	S-Methyl propanethioate (CAS num: 5925-75-7)	0.13 ± 0.02	801	785	88	Thiocarboxylic acid
	1-Undecene (CAS num: 821-95-4)	0.43 ± 0.02	1092	1087	98	Alkene
Penicillium strain ks20_f15	trans-Nerolidol (CAS num: 40716-66-3)	1.34 ± 0.26	1570	1564	95	Sesquiterpenoid
Penicillium strain ks20_f18	cis-Thujopsene (CAS num: 470-40-6)	2.60 ± 0.04	1445	1426	95	Sesquiterpenoid
	beta-Chamigrene (CAS num: 18431-82-8)	2.17 ± 0.12	1492	1478	96	Sesquiterpenoid
	beta-Himachalene (CAS num: 1461-03-6)	1.27 ± 0.05	1514	1500	91	Sesquiterpenoid
	alpha-Chamigrene (CAS num: 19912-83-5)	1.19 ± 0.02	1519	1507	96	Sesquiterpenoid
	beta-Cedrene (CAS num: 546-28-1)	0.10 ± 0.00	1435	1424	92	Sesquiterpenoid
	4-Methyl-3-hexanone (CAS num: 17042-16-9)	0.94 ± 0.06	842	801	97	Ketone
	3-Methylanisole (CAS num: 100-84-5)	0.10 ± 0.00	1024	1029	97	Benzenoid
	Bisabolene (CAS num: 495-62-5)	0.45 ± 0.09	1487	1509	92	Sesquiterpenoid
Penicillium strain ks20_f54	3-Octanone (CAS num: 106-68-3)	7.64 ± 1.30	989	988	98	Ketone
	1-Octen-3-ol (CAS num: 3391-86-4)	2.56 ± 0.68	983	982	99	Fatty alcohol
	alpha-Gurjunene (CAS num: 489-40-7)	1.17 ± 0.26	1472	1408	89	Sesquiterpenoid

	beta-Myrcene (CAS num: 123-35-3)	0.69 ± 0.06	993	981	95	Monoterpene
Penicillium strain ks20_f10	3-Octanone (CAS num: 106-68-3)	0.73 ± 0.14	992	988	97	Ketone
	beta-Ocimene (CAS num: 13877-91-3)	0.94 ± 0.18	1054	1050	98	Monoterpene
	4-Methyl-2-hexanone (CAS num: 105-42-0)	0.43 ± 0.01	853	846	93	Ketone
	cis-Thujopsene (CAS num: 470-40-6)	0.41 ± 0.02	1449	1426	97	Sesquiterpenoid
Penicillium strain ks20_f14	beta-Himachalene (CAS num: 1461-03-6)	2.54 ± 0.07	1519	1500	92	Sesquiterpenoid
	alpha-Chamigrene (CAS num: 19912-83-5)	1.69 ± 0.27	1453	1507	88	Sesquiterpenoid
	cis-Thujopsene (CAS num: 470-40-6)	1.56 ± 0.04	1449	1426	95	Sesquiterpenoid
	Bisabolene (CAS num: 495-62-5)	1.34 ± 0.02	1483	1509	91	Sesquiterpenoid
	Phenol (CAS num: 108-95-2)	0.31 ± 0.07	993	950	92	Benzenoid
	beta-Cedrene (CAS num: 546-28-1)	0.43 ± 0.01	1439	1424	92	Sesquiterpenoid
Penicillium strain ks20_f19	Methoxybenzene (CAS num: 100-66-3)	1.43 ± 0.13	923	912	96	Benzenoid
	3-Octanone (CAS num: 106-68-3)	0.95 ± 0.29	992	988	97	Ketone
	Butylated hydroxytoluene (CAS num: 128-37-0)	0.92 ± 0.14	1525	1514	96	Benzenoid
Penicillium strain ks20_f30	Methoxybenzene (CAS num: 100-66-3)	3.49 ± 0.22	923	912	96	Benzenoid
	3-Octanone (CAS num: 106-68-3)	1.32 ± 0.02	992	988	97	Ketone
	1-Octen-3-ol (CAS num: 3391-86-4)	1.28 ± 0.07	986	982	95	Fatty alcohol
	Butylated hydroxytoluene (CAS num: 128-37-0)	0.20 ± 0.03	1525	1514	95	Benzenoid
	trans-Nerolidol (CAS num: 40716-66-3)	0.74 ± 0.05	1574	1564	94	Sesquiterpenoid
	1,3,5-Heptatriene (CAS num: 17679-93-5)	0.35 ± 0.03	787	781	96	Alkene

Penicillium strain ks20_f52	D-Limonene (CAS num: 5989-27-5)	10.85 ± 0.48	1034	1031	96	Monoterpenoid
	Isoborneol (CAS num: 124-76-5)	10.81 ± 1.41	1167	1160	97	Monoterpenoid
	beta-Pinene (CAS num: 18172-67-3)	2.30 ± 0.07	981	964	96	Monoterpenoid
	alpha-Terpinolene (CAS num: 586-62-9)	2.32 ± 0.12	1094	1090	97	Monoterpenoid
	alpha-Pinene (CAS num: 80-56-8)	1.07 ± 0.02	938	939	98	Monoterpenoid
	alpha Phellandrene (CAS num: 99-83-2)	0.68 ± 0.03	1010	996	86	Monoterpenoid
	Methoxybenzene (CAS num: 100-66-3)	0.60 ± 0.08	925	912	94	Benzenoid
	alpha-Terpineol (CAS num: 10482-56-1)	0.23 ± 0.02	1201	1187	94	Monoterpenoid
	Camphene (CAS num: 79-92-5)	6.83 ± 0.24	953	933	97	Monoterpenoid
P. agathidicida strain NZFS3770	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (CAS num: 6846-50-0)	0.35 ± 0.03	1602	1587	80	Carboxylic acid
	2,5-di-tert-Butyl-1,4-benzoquinone (CAS num: 2460-77-7)	0.42 ± 0.06	1475	1466	89	Carbonyl compound
	Methyl salicylate (CAS num: 119-36-8)	0.28 ± 0.07	1203	1193	93	Benzenoid
	4-ethylphenol (CAS num: 123-07-9)	0.28 ± 0.02	1176	1142	94	Benzenoid
	2-Phenylethanol (CAS num: 60-12-8)	0.65 ± 0.05	1122	1116	97	Benzenoid

Table D.4. The accepted volatile organic compounds detected in the fungal and bacterial strains analysed. The molecular weight, molecular formula and literature linear retention index (LRI) of each compound is shown, including the associated reference selected as the literature source for the LRI value.

CAS number	Compound Name	Molecular weight	Molecular Formulae	Literature LRI	Reference
15192-80-0	Octa-2,4,6-triene	108	C8H12	925	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a
586-62-9	alpha-Terpinolene	136	C10H16	1090	(Oliveira et al., 2007)
546-28-1	beta Cedrene	204	C15H24	1424	(Champagnat et al., 2006)
123-35-3	beta Myrcene	136	C10H16	981	(Kartal et al., 2007)
13877-91-3	beta Ocimene	136	C10H16	1050	(Juliani & Simon, 2002)
17679-93-5	1,3,5-Heptatriene	94	C7H10	781	(Pino et al., 2005)
6874-10-8	alpha Ocimene	136	C10H16	1052	(J. Liu et al., 2006)
99-83-2	alpha Phellandrene	136	C10H16	996	(Kartal et al., 2007)
4045-44-7	Pentamethylcyclopentadiene	136	C10H16	983	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a
40716-66-3	trans-Nerolidol	222	C15H26	1564	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a
1461-03-6	beta-Himachalene	204	C15H24	1500	(Mevy et al., 2006)
489-40-7	alpha-Gurjunene	204	C15H24	1408	(Hazzit et al., 2006)
3391-86-4	1-Octen-3-ol	128	C8H16O	982	(Sharififar et al., 2007)
821-95-4	1-Undecene	154	C11H22	1087	(Beens et al., 1998)
6846-50-0	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	286	C16H30	1587	(Andriamaharavo, 2014)
79-92-5	Camphene	136	C10H16	933	(Kartal et al., 2007)
2460-77-7	2,5-di-tert-Butyl-1,4-benzoquinone	220	C14H20	1466	(Custer, 2009)
110-43-0	2-Heptanone	114	C7H14O	889	(Santos et al., 1998)

105-42-0	4-Methyl-2-hexanone	114	C7H14O	846	(Xu et al., 2003)
821-55-6	2-Nonanone	142	C9H18O	1091	(Adams, 2000)
2345-28-0	2-Pentadecanone	226	C15H30	1698	(Schwob et al., 2004)
80-56-8	2-Pinene	136	C10H16	939	(Hazzit et al., 2006)
0-00-0	3-Chloropropionic acid, 4-isopropylphenyl ester	226	C12H15	1544	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a
17042-16-9	4-Methyl-3-hexanone	114	C7H14O	801	(Paolini et al., 2006)
0-00-0	2-butan-2-yl-3-methoxy-5-(2-methylpropyl) pyrazine	222	C13H22N2O	1463	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a
106-68-3	3-Octanone	128	C8H16O	988	(Jean et al., 1993)
0-00-0	4-Chlorobutyric acid, 4-isopropylphenyl ester	240	C13H17	1732	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a
59744-12-6	7-Oxabicyclo[4.1.0]heptane, 1-(2,3-dimethyl-1,3-butadienyl)-2,2,6-trimethyl-, (E)-	220	C15H24	1461	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a
100-84-5	3-Methylanisole	122	C8H10O	1029	(Jalali-Heravi & Garkani-Nejad, 1993)
100-66-3	Methoxybenzene	108	C7H8O	912	(Hoskovec et al., 2005)
18172-67-3	beta-Pinene	136	C10H16	964	(Kartal et al., 2007)
116-53-0	2-Methylbutanoic acid	102	C5H10O	894	(Alissandrakis et al., 2007)
128-37-0	Butylated hydroxytoluene	220	C15H24	1514	(Adams et al., 2005)
470-40-6	cis-Thujopsene	204	C15H24	1426	(Skaltsa et al., 2003)
3658-80-8	Dimethyl trisulfide	126	C2H6S3	971	(Pham et al., 2008)
5989-27-5	D-Limonene	136	C10H16	1031	(Jordan et al., 2002)
124-76-5	Isoborneol	154	C10H18O	1160	(Baranauskienė et al., 2003)
10482-56-1	alpha-Terpineol	154	C10H18	1187	(Skaltsa et al., 2003)
20333-39-5	Ethyl methyl disulfide	108	C3H8S2	833	(Misharina & Golovnya, 1989)
7289-53-4	1-Methoxyundecane	186	C12H26O	1290	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a
2855-19-8	2-Decyloxirane	184	C12H24	1307	(Yayli et al., 2006)

108-95-2	Phenol	94	C6H6O	950	(Berezkin et al., 1997)
96-76-4	2,4-Di-tert-butylphenol	206	C14H22O	1513	(Verekin et al., 1990)
123-07-9	4-ethylphenol	122	C8H10O	1142	(Engewald et al., 1988)
60-12-8	2-Phenylethanol	122	C8H10O	1116	(Alissandrakis et al., 2007)
13360-64-0	2-Ethyl-5-methylpyrazine	122	C7H10N2	1000	(Solina et al., 2005)
5925-75-7	S-Methyl propanethioate	104	C4H8OS	785	(Garbuzov et al., 1985)
18431-82-8	beta-Chamigrene	204	C15H24	1478	(Asuming et al., 2005)
19912-83-5	alpha-Chamigrene	204	C15H24	1516	(Asuming et al., 2005)
5756-24-1	Dimethyltetrasulfane	158	C2H6S4	1200	(Misharina & Golovnya, 1989)
495-62-5	Bisabolene	204	C15H24	1509	Mass Spectrometry Data Center https://www.nist.gov/srd/nist-standard-reference-database-1a

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